Analysis of Polysaccharides using Matrix Assisted Laser Desorption/ Ionization Time-of -flight Mass Spectrometry (MALDI-TOFMS)

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ABSTRACT

This project focuses on the development of matrix-assisted laser desorption / ionization mass spectrometry (MALDI) method for the analysis of polysaccharides. Polysaccharide is a class of natural polymer widely applied in pharmaceutical industry. Because of the various experimental factors, including sample preparation and detector sensitivity, direct use of MALDI-MS method for characterization of dispersed polymers, such as polysaccharides, has not been achieved.

Different physical and chemical properties of polysaccharides, such as acid-liability, polarity, mass range and polydispersity, have been found to limit the applicability of mass spectrometry based methods for their characterizations. In the present study, a survey study of effective matrix systems for polysaccharide analysis was conducted. Since MALDI matrices are commonly acidic in nature, particular attention was given to the use of basic co-matrix systems for reducing acid-catalyzed fragmentation of the polysaccharide. Despite the success in developing effective matrix systems for analysis of dextran (1→6 linked polysaccharides) samples (M_w 1,000, 5,000, 12,000), the observed distributions of the various polymer components were found to differ significantly from the values obtained by gel permeation chromatography (GPC) method. Using different sample preparation methods for measuring blended mixtures of two narrow distributed dextran samples of different masses, it was concluded that on-probe fractionation plays an important role in causing the shift in the molecular weight distribution under conventional drop drying sample preparation method. This on-probe fractionation was tentatively attributed to the difference in the solubility between the low-mass and high-mass components for dispersed polysaccharides. As differential solubility is an intrinsic physical property of homologues, the use of liquid matrix system was explored. Since liquid matrix systems avoid crystallization process, any discrepancies arising from the on-probe fractionation would be alleviated. More consistent results were obtained in comparing to the GPC values for dextran samples analyzed.

摘要:

本論文主要研究基質輔助激光解吸電離質譜(MALDI)方法在多糖分析方面的開發應用,多糖是一類廣泛應用於製藥工業的天然高分子。由於不同的實驗因素,包括樣品的製備和檢測器的敏感性,直接用基質輔助激光解吸電離質譜方法檢定分散的高分子,如多糖,未能成功。

多糖不同的物理和化學性質,如酸度、極性、分子量範圍和多分散性,限制了質譜方法對它們的檢定。在目前的研究中,進行了有效的基質系統對多糖分析的全面研究。因為基質通常都是酸性的,所以特別注意應用鹼性助基質系統以減輕酸催化多糖裂成碎片。雖然成功開發有效的基質系統分析葡聚糖樣品(1→6連接的多糖),我們發現從質譜得到分子成份的分佈明顯不同於膠滲透方法(GPC)得到的結果。用不同的製備方法測量兩個窄分佈的葡聚糖樣品的分子量,得到的結果是:製備過程中分級分離在常規液滴乾燥樣品製備方法中引起分子量分佈移動起重要的作用。這種製備過程中分級分離歸因於低分子量和高分子量分散性多糖成分之間不同的溶解性。因為不同的溶解性是同系物中固有的物理性質,所以我們應用了液體基質系統。由於液體基質系統避免了結晶過程,所以任何由製備過程中分級分離引起的差異都會緩和。在同膠滲透方法方法比較對葡聚糖樣品分析中,得到了一致性更高的結果。

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ABBREVIATIONS

α-CCA	α-Cyano-4-hydroxy-cinnamic acid
2,5-DHB	2,5-Dihydroxybenzoic acid
2-NPOE	2-Nitrophenyl octyl ether
3-AP	3-Aminopyridine
3-AQ	3-Aminoquinoline
3-NBA	3-Nitrobenzyl alcohol
ABA	Aminobenzyl alcohol
ACTH	Adrenocorticotropic hormone fragment 18-39
ATT	6-Aza-2-thiothymine
CTMA	Cetyl trimethyl ammonium bromide
Da	Daltons
DMAP	3,3-Dimethylaminopyridine
Gly	Glycerol
GPC	Gel permeation chromatography
HABA	2-(4-Hydroxyphenylazo) benzoic acid
HPA	3-Hydroxypicolinic acid
IAA	3-Indoleacrylic acid
K.E.	Kinetic energy
LD	Laser desorption
m	Mass of a particle
m/z	Mass-to-charge ratio
MALDI	Matrix-assisted laser desorption/ ionization

CHAPTER ONE

INTRODUCTION

1.1 Carbohydrates

Among the four major classes of natural products, carbohydrates predominate in quantitative terms. Carbohydrates constitute an important class of molecules in biological systems. It widely occurs in plant kingdom, algae, bacterial, fungal and also higher animals. Besides, it can also be covalently lined to with proteins and lipids to form glycoproteins and glycolipids. This class of molecules plays significant roles in living system and the development of culture by providing clothing, food, shelter and writing materials.

Historically, carbohydrate is a collective term referring to the "hydrate of carbon" with a general of formula $C_n(H_2O)_n$. However, it was found that carbohydrate contains both hydroxyl groups and carbonyl groups; and is actually polyhydroxyl aldehydes or ketones. Carbohydrate analysis is complex because of its diverse variations. Variations of carbohydrate is originated from the existence of various monosaccharide units and their anomers, glycosidation sites and the various positions and extent of branching. All these configurations play important roles in affecting the morphology and the properties of carbohydrate chains and hence their applications. Homopolysaccharide consisting of glucose monomeric unit is called a *glucan*. It is one of the polysaccharides that have been applied in ancient times. Monosaccharide units linked at varied sites, degree of branching, and anomers have composed different important polysaccharides, including starch, glycogen, cellulose, and dextran.

Starch is usually found in tissue parts of organs and serves as media for energy storage. Corn, tapioca, potato and sago are the most representative sources of starch. Starch is composed of 10-20% amylose and 80-90% amyopectin. Amylose is a linear α-D-(1→4)-linked glycan and tends to have a compact helical arrangement. Typical molecular weight ranges from 150,000 to 600,000 Daltons. Amylopectin has a highly branched structure with α-D-(1→6) branch points. Typically, it has a molecular weight of 1 to 6 million Daltons. Because of the high viscosity and the gelatinization properties, starch is generally applied in industry as dusting agent, swollen starch granule, addition in manufacture of paper products, as a flocculent and an aid in pigment retention, dried film as in paper coating or in sizing of textiles fibers.

Similar to amylopectin, the structure of glycogen consists of α-D-(1→4)-linked glycan with additional α-D-(1→6) branch points. Glycogen can be found in muscle, skeleton, livers and brains of animals; and in the tissues of invertebrates, bacteria and protozoa. Molecular weight of glycogen is as high as 100 million Daltons. These macromolecules are usually too large to diffuse across the cell membrane and remain inside the cell to serve as energy reserve. These molecules also play an important role in maintaining the osmotic pressure of the cell and preventing rupture of the cell membrane.

Cellulose is a pure homopolysaccharide with exclusively unbranched β-D-(1→4)-linked glucose units. The β-linked configuration of the anomeric carbon atoms makes cellulose chains essentially linear. The linear chain configuration is stabilized

through strong intermolecular hydrogen bonds and allows a tight packing of several, anti-parallel chains. This assembly of cellulose molecules gives a highly insoluble, rigid and fibrous polymer and forms the basis of vegetable fibers. Cellulose always occurs as a crystalline array of many parallel, oriented chains of microfibrils. The glycan chain length (degree of polymerization) varies from about 2,000 up to > 25,000 glucose residues. The size of the microfibril varies from 36 chains to 1200 chains. Because of the interesting features such as sound transducing and absorptive properties of microbial cellulose, cellulose has been used in manufacturing of audio-components, health foods, and wound care products.

Dextran is a kind of homopolysaccharide of glucose, synthesized by bacterial enzyme or cell-free bacterial culture such as Leuconostocs. In dextran molecules, glucose are linked via 95% of α (1→6) linkages and the rest α (1→2), α (1→3) or α (1→4) linkages. The morphology and the conformation of the polysaccharide molecules rely on the degree of cross-linking among the different polysaccharide chains. Highly cross-linked polysaccharides usually have gel-type morphology and have been widely used as molecular sieves. Typical example is the "Sephadex". It is a packing material for size-exclusion chromatography (SEC). Different molecular exclusion limits are obtained by using polysaccharides with different degree of cross-linking. It is widely used to segregate the macromolecules, such as polysaccharide, according to their sizes. Besides, it can also serve as solid support for affinity chromatography in which the desired-affinity ligand is covalently coupled to the polysaccharide surface.

Apart from being energy storage and supporting materials, carbohydrates and their conjugates also participate in metabolism and immuno-modulating activities[1,2]. The sulfated polysaccharide[3] and dextran sulfates[4] have been shown to suppress the activities of the Human Immunodeficiency Virus (HIV).[5,6] HIV is the causative agent of the Acquired ImmunoDeficiency Syndrome (AIDS). Carbohydrates components of glycoconjugates [7] specify the biological recognition that is particularly important in cell-cell interactions that influence growth, differentiation, formation of organs, fertilization, processes of bacterial and viral infection, formation of tumors, tumor metastasis and the prevention of autoimmune reactions. Their medical applications are exemplified by anti-tumor and anti-viral drugs[8]. Typical examples include polymers of glucose via β (1→3) linkages (curdlan) and α(1→6) linkages (dextran). In many cases, molecular weight (MW) and molecular weight distribution (MWD) constitute important parameters in determining the application of polysaccharides. For instance, dextran molecules of suitable sizes (~ 70,000 Daltons) are the basis of one of the common therapeutic agents for restoring blood volume for mass casualties, preventing deep venous thrombosis by increasing the osmotic load. In the presence of dextran molecules, an osmotic pressure was found to develop at the membranes of blood capillaries. Water was then reabsorbed into the blood to restore the osmotic pressure of the plasma. The duration of the intravascular osmotic pressure properties was found to show a good correlation with the molecular weight of the dextran molecules. Small dextran molecules (<< 70,000 Da) were found to escape rapidly from the body through the renal system. Although larger dextran molecules can give a more sustainable effect, these molecules become antigenic as their molecular weights exceed 90,000 Daltons.

1.2 Impact of molecular weight of polysaccharides

Molecular weight (MW) and molecular weight distribution (MWD) of polysaccharides constitute a controlling factor in determining their physical properties. Therefore, carbohydrates with different MWs can be applied in different industries. In the application of carbohydrates in medical science, attention must be paid on the MW of the carbohydrate used. Large dextran molecules can stay inside the body to maintain the osmotic pressure. These molecules, however, become antigenic as their molecular weights exceed the limit. Since the pioneer study of dextran by Elvin A. Kabel. in 1952, the antigenic property has been recognized[9,10] as its molecular weight above 90,000 Daltons.

Besides, dextran sulfate with molecular weight 10,000 Daltons are active in anti-HIV formulations. Higher molecular weight dextran sulfate is, however, toxic because it precipitates fibrinogen resulting in embolism of the blood vessels. On the other hand, its toxicity is greatly reduced by a drastic reduction in the molecular weight to 20,000 Daltons, which approximates the molecular weight of heparin (17,000 Daltons). Therefore, molecular weight monitoring is a vital procedure for using polysaccharides as fine chemicals or drugs.

1.3 Molecular weight determination of polysaccharide

Common methods for measuring molecular weight and molecular weight distribution of polymers have their strengths and weaknesses. Laser scattering and gel premeation chromatography are by far the most common methods for analysis of

polysaccharides and other polymers. More recently, mass spectrometry has been shown to provide accurate structural and molecular weight information for various polymers.

1.3.1 Laser Scattering

Laser Scattering is a technique widely used to characterize polymeric materials[11,12]. This method applies the principle of "light scattering" with laser as a light source[13]. When a laser beam is directed through a dilute analyte solution, each particle in solution undergoes scattering independently with no significant interactions. In the laser light scattering measurement, intensities of scattered light are measured as a function of the viewing angles; and the measurement is repeated using sample solution of different concentrations. The result is summarized by using the Zimm plot. A graph K_c/R_θ is plotted against $\sin^2(\theta/2)+bc$, where K_c is the optical constant, R_θ is the scattering intensity at angle θ, c is the weight of particles per unit of particles per unit volume and b is an arbitrary constant. By extrapolation, the intensity at zero angle at infinite dilution was recorded. Although the measurement of this type gives information about the weight average molecular weight (M_w), it also relates to the size and shape of the macromolecules in solution and sometimes provides interesting information on the interactions between the solvent and polymer molecules.

Since the laser scattering method measures the MWD of the polymer by analyzing its physical properties, no separation is needed. The sample can be analyzed directly with simple operation technique. This technique is more suitable for high molecular

weight samples because the error related to refractive index is negligible as the molecule weight increases. However, this technique is sensitive to the presence of dust in the sample and the formation of solution aggregates; and gives unreliable result. Therefore purification of sample is crucial, thus complicating the analytical procedure and lengthening the total time for analysis.

1.3.2 Gel Permeation chromatography (GPC)

Gel Premeation chromatography (GPC) sorts and separates components of a sample according to their hydrodynamic volumes. Various detectors, including ultraviolet (UV), refractive index (RI) detector and low-angle laser light scattering (LALLS), have been used in conjunction with the GPC. In typical GPC analysis of biological samples, a gel of high molecular weight and highly cross-linked polysaccharide is used as the stationary phase, whereas water is normally used as the mobile phase. Depending on the degree of cross-linking, the stationary phase has different pore sizes to accommodate molecules of different hydrodynamic volumes. During an elution process, the extent of permeation of a molecule within the pores governs the time of retention of the molecules. Since different molecules have different hydrodynamic volume (i.e. molecular size and shape), separation of the various components of a sample can thus be achieved through their differential retention within the stationary phase. Molecules that are too large to enter any of the pores within the gel will be totally excluded and have the shortest elution time. Molecules that are small enough to permeate into every pore of the gel will be totally permeated and have the longest elution time. For molecules that have sizes comparable to the sizes of the pores, the degree of permeation (and hence the retention time) depends

critically on their hydrodynamic volumes. For separation of polymeric materials, such as polysaccharides, the hydrodynamic volume relates linearly to the molecular weight of the molecules due to their similar solution conformations in a specific solvent system. Therefore, the molecular weight and molecular weight information of polysaccharides can be obtained from the retention time and the distribution of the retention time, respectively.

Although Gel Permeation Chromatography (GPC) is a widely accepted method for measurement of molecular weight information for polymers, it suffers however from poor resolution, long analysis time and absence of suitable reference compounds for calibration. In addition, this separation-based method provides no information regarding the mass of the repeating unit and the end group structure. For analysis of polysaccharides, GPC method also requires the use of relatively insensitive detector, such as refractive index detector, due mainly to the absence of any UV/visible chromophores. In many instances, the combining impacts of low detector sensitivity and the limited solubility of the sample in water have rendered the analysis of high-mass and/or high degree of cross-linkage polysaccharides impossible.

1.3.3 Mass Spectrometry

Conventional mass spectrometry analysis involves the use of an energetic beam of electrons to ionize analyte molecules in gas phase. The electron-impact ionization method (EI) requires the analyte molecules be either volatile (in vacuum conditions) or thermally stable. Because of the large molecular size, the presence of a large number of hydrogen bonds and thermally labile hydroxyl groups, polysaccharides molecules are intrinsically involatile and thermally labile and are therefore

unsuitable for analysis by EI-MS. Similarly, chemical ionization method (CI) is also not amendable for polysaccharides analysis.

During the past decades, a number of "soft" desorption/ ionization methods have been developed. These methods including field desorption (FD),[14] thermal ionization (TI),[15] fast atom bombardment (FAB),[16] secondary-ion mass spectrometry (SIMS), plasma desorption (PD),[17] and laser desorption (LD).[18,19] These methods are more compatible for analysis of involatile and thermally liable compounds. Nevertheless, these methods were only capable of analyzing relatively low-mass molecules. An upper mass limit of 5,000 to 10,000 Daltons has been found. More recently, the independent development of electrospray ionization (ESI) and matrix-assisted laser desorption / ionization (MALDI) techniques have made direct mass spectrometry analysis of large biomolecules feasible. Intact biomolecules with masses up to hundreds of thousand Daltons have been desorbed and ionized. When the ESI and MALDI methods are compared, the ESI method is generally considered to be less useful for determination of the molecular weight and molecular weight distribution for polymers due presumably to the overlapping of the peaks of the oligomers at different charge states. In addition, the success of the ESI analysis also depends on the availability of suitable solvents that can dissolve a substantial amount of polymer and can be sprayed under normal ESI conditions.[20]

The MALDI method is, on the other hand, more compatible to different solvents and produces mainly singly-charged ions (occasionally doubly-charged ions). It is therefore more suitable for analysis of highly complex mixtures such as polymers.

1.4 Matrix assisted laser desorption/ ionization

1.4.1 Laser desorption

The use of laser photons to assist desorption and ionization of involatile and thermal liable compounds can be traced back to early 1970's.[21,22,23,24,25]. Lasers emitting photons of various wavelengths, ranging from vacuum-ultraviolet (UVU) up to infrared (IR), have been used in mass spectrometry. Both continuous and pulsed lasers have also been used. Depending on the wavelength of the laser and the duration of the laser beam, different mechanisms of desorption / ionization have been postulated. Pulsed lasers have generally been found to be more useful in desorption and ionization of organic molecules. The absorption coefficient of the analyte molecules at the wavelength of the laser photons has been found to influence the success of the laser desorption measurement. For samples with little or not absorption at the laser wavelength, extensive fragmentation of the analyte molecules usually occurs. Intact molecule-ions larger than 700 Daltons could not normally be observed. For samples with high absorption coefficient at the wavelength of the laser, intact molecule-ions as large as 1,000 Daltons could be observed under suitable laser fluence. All attempts to analyse larger molecules (>2,000 Daltons) was however failed under direct laser desorption conditions.

1.4.2 Matrix-assisted laser desorption / ionization

Matrix-assisted laser desorption/ ionization mass spectrometry[26,27] (MALDI-MS) was first introduced in 1988. It is now one of the powerful analytical techniques for analysis of biomolecules including oligonucleotides[28], carbohydrates[29,30], and

proteins[31]. In comparison to the common methods used in biomedical sciences for measuring molecular weight, i.e. sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)[32,33], the MALDI method offers several advantages, such as shorter analysis time, higher sensitivity and higher precision of measurement. Although the accessible mass range of MALDI method remains inferior to SDS-PAGE, protein molecules with masses up to several hundred kilo-Dalton[34,35] have been successfully analyzed at picomole to femtomole level[36,37,38,39].

The main differences between conventional laser desorption methods and MALDI technique is the use of the "*matrix*" materials. The roles of the matrix in the desorption and ion formation processes have widely been discussed.[40,41,42,43] It is a general consensus that it absorbs laser irradiation and leads to the breakup of a microvolume of the solid materials. Besides, it prevents association or aggregation among sample molecules. It has been postulated that potential matrix materials should carry the following properties: (a) it should have a reasonable high molar extinction coefficient at the wavelength of the laser; (b) it should dissolve in solvent(s) that is/are miscible with that of analyte solution; (c) it should have relatively low heat of sublimation, good vacuum stability and low reactivity with the analyte molecules. In order to be an efficient matrix, it must also be able to generate a high yield of ions from the desorbed analyte molecules.[44] Even though many studies have been conducted on the properties of the matrix materials, discovery of new and effective matrices is still done by trial-and-error.

Many investigations have been focused on the study of the process(es) of desorption and ionization under MALDI conditions. Different hypotheses have been developed

to explain different aspects of the MALDI processes. In the "cool plume model"[45,46], the scenario about the heating and phase transition by laser energy was outlined. After energy is deposited onto the matrix, the surface temperature is elevated above the sublimation temperature of the matrix. The matrix molecules are thus sublimed to gas phase. The plume undergoes a gas dynamics expansion and the embedded analyte molecules are desorbed together. Similarly, the "pressure pulse model" [47] proposed the development of a pressure gradient along the depth of the solid sample through the absorption of the penetrating laser photons. As the pressure exceeds some critical values, the surface layer ruptures. Such molecular expansion desorbs the embedded analyte molecules. In the "Desorption Induced by Electronic Transition" (DIET) model, [48, 49] the absorbate (matrix) is excited by photons electronically leading to an anti-bonding state or ionized state. As it is a non-equilibrium process, the excited states are usually in repulsive range of interaction potential. Then, the excited particle will experience a repulsion field and leave the surface with some kinetic energies.

To explain the low degree of fragmentation for the analyte molecules, it was proposed that there is a "bottleneck" in which energy transfer between the "hot" matrix molecules and the "cold" analyte molecules is exceptional slow. The Homogeneous Bottleneck (HB) model proposed that such inefficient energy redistribution was due presumably to the structural mismatch between the matrix and analyte molecules. Since the desorbed analyte molecules remain internally cool and are therefore not prompt to decomposition. This HB model is further supported by classical molecular dynamics simulation and kinetic models. In the simulation, the

analyte molecules/ ions were found to have considerately lower temperature than the solid surface provided that the surface heating or sublimation rate is rapid enough. In addition, it is also believed that the expansion of the desorption plume might actually cool the plume to very low temperature, i.e. < 200K. Such cooling effect might further stabilize the entrained larger biomolecules ion against decomposition.

From the experiments with molecular thermometer, the possibility of having thermal ionization has been eliminated. The generally accepted channel of molecular ion formation is chemical in nature, such as proton transfer and adduct-ions formation. Gimon and co-worker have suggested that the formation of analyte ions results from proton-transfer reactions between the electronically excited matrix molecule and the co-desrobed analyte molecules. Since matrix materials with and without acidic groups were reported effective for MALDI experiments, simple acid-base reaction between matrix and analyte could not be used to explain the ionization process(es) under MALDI conditions. Proton-transfer reactions between analyte and electronically (for UV-laser) or ro-vibrationally (for IR-laser) excited matrix molecules appear to be more promising in explaining ionization process(es) in MALDI.

1.5 MALDI-TOFMS analysis of polymers

Conventionally, gel permeation chromatography (GPC) is one of the major instrumental methods for the determination of the molecular weight of polysaccharides. It has however suffered from many limitations. The accuracy of the GPC measurement is not good because of the peak broadening effect and the

absence of suitable reference compounds for calibration.[50] It is also a time consuming process. In addition, because of the absence of any UV/visible chromophores in common polysaccharide samples, the rather insensitive refractive index (RI) detector is usually employed instead of UV detector. Since the introduction of MALDI-TOFMS in 1988, it has undergone rapid development in the areas of biomolecule analysis.

Despite of the success in the analysis of protein samples, MALDI technique was found to provide inconsistent information for polysaccharides (i.e. dextran) as compared with those obtained from gel permeation chromatography (GPC)[51,52,53]. It was found that the measurement discrepancies increased proportionally to the average molecular weights of the dextran samples. Very often, the molecular weight distributions were heavily distorted towards the low-mass side of the spectrum. Normal distribution was not normally obtained.[54] The reliability of MALDI-TOFMS in molecular weight distribution determination has been evaluated previously by analyzing narrow distributed polystyrene standards[55]. The results proved that MALDI is a reliable and accurate method for MWD determination if the polydispersity of the polystyrene sample is smaller than 1.3.[56,57,53,58] By contrasting the results of the narrow distribution polymer standards and that of the dispersed samples, polydispersity (P.D.) is believed to be a key factor leading to the failure in the MALDI analysis of dispersed samples. Although an empirical correlation has been established between the success/failure of MALDI analysis and the polydispersity of the sample, the underlining reason(s) for such correlation is still uncertain. Factors involved in the sample preparation methods,[59,60,61,62,63]

instrumental arrangement and the methods of data processing[64,65,66] have previously been evaluated.

In 1995, Garrozzo D. *et al*[54] fractionated a polysaccharide sample by using low pressure GPC on a Sephedax column. The fractionated samples were then analyzed by MALDI-TOFMS. The spectra of narrow distribution mass fractions with masses up to 90,000 Daltons were successfully recorded. Based on the experimental result, it was proposed that lighter components are preferentially desorbed and ionized, and/or heavier components are suppressed, in MALDI processes.

In attempts to overcome the problems associated with the MALDI analysis of dispersed samples, much effort has been devoted to the development of on-line and/or off-line GPC and MALDI-TOFMS methods[67,68,69,70,71,72] for the corrected evaluation of MW and MWD of dispersed polymers.

1.6 Outline of the present work

The main objective of the project is to evaluate the possibility of using MALDI-TOFMS for the measurement of the MW and MWD of dispersed polysaccharide. Chapter one gives an overview of the MALDI mass spectrometry and the research background on the polysaccharide analysis. Chapter two covers the instrumentation and experimental methods used in the present study. In Chapter three, the effect of co-matrix systems on the MALDI analysis of polysaccharide was investigated. Results concerning the effect of the modified matrix system on analysis of different molecular weight dextran samples are discussed. In an attempt to explain the measurement discrepancies between GPC and MALDI-MS for analysis of dispersed

polysaccharides analysis, Chapter four investigates the possible formation of a molecular gradient across the thickness of the sample crystals. Chapter five focuses on the development of liquid matrix systems for analysis of dispersed dextran. The concluding remark of the project is finally summarized in Chapter Six.

CHAPTER TWO

INSTRUMENTAL AND EXPERIMENTAL

2.1 Matrix-assisted laser desorption/ ionization Time of flight Mass Spectrometry (MALDI-TOFMS)

Matrix-assisted laser desorption/ ionization (MALDI) is a pulsed ionization source. A packet of ions is generated by a short laser pulse. Time-of-flight (TOF) analyzer is an instrument that measures the flight time of ions of the same kinetic energy through a long field free tube. Ions of different mass-to-charge ratios will take different time to transverse the tube. In MALDI-TOF configuration, ions are generated in the ion source region and then transverse the flight tube to arrive at the detector. The main drawback of the MALDI-TOFMS is limited resolution. Parameters limiting the resolving power of a MALDI-TOF instrument include spatial distribution, initial kinetic energy distribution and temporal distribution of ions. The contribution of these parameters on the time broadening of the ion packet can be reduced by using different instrumental modifications in the ion source region and field free region.

Figure 2.1 : A picture of the MALDI-TOFMS.

2.2 Delayed extraction

It is a modification applied in ion source region. Delayed extraction technique uses a pulsed, time-delayed two-stage extraction potential to avoid the undesired initial conditions[73]. Time dispersion caused by the initial desorption kinetic energies of the MALDI generated ions and the spatial distributions of the molecules during the ion formation process are alleviated.

2.3 Time of flight mass spectrometry (TOFMS)

The principle of TOF is simple and direct. It separates ions with different mass to charge ratio by their respective time-of-flight (TOF). The resolution of time of flight is limited but it is still very popular and universal. Apart from simple instrumentation, there are many advantages over the other techniques including simple operation and short analysis time, in microseconds. Secondly, it does not have an upper mass limit. Thirdly, its transmission efficiency and hence the sensitivity is high. Finally, the instrument is inexpensive.

2.3.1 Linear time-of-flight mass spectrometry

In TOF analysis, ions are formed in the source region and an electric field (E) is applied across the source (s) to accelerate the ions into the field free region with defined kinetic energy (K.E.)

$$K.E. = zeEs$$

where z is number of charge and e is the charge on an electron. When ions pass through the extraction grid, their terminal velocities relate inversely proportional to the square root of their mass.

$$v = (2zeEs/m)^{½}$$

For a fixed field free tube of distance D, their TOF measured is

$$t = (m/ 2zeEs)^{½}D$$

Correlating the two equations, the relationship between time and mass-to-charge ratio is:

$$m/z = 2eEs\ (t/D)^{½}$$

The flight time is recorded when ions strike onto the microchannel plate located at the end of the flight tube. In a linear TOF instrument, the path that ions pass in the flight tube is a straight line. In simple linear TOFMS, mass resolution is relatively poor due presumably to the initial kinetic energy distribution of the MALDI ions. In a more sophisticated instrument, an energy-compensating device, such as reflectron mirror, is sometimes employed.

2.3.2 Reflectron

Reflectron is an energy-compensating device, which corrects the initial kinetic energy distribution of the MALDI-generated ions. It compensates the energy distribution by adjusting the flight paths for ions of the different kinetic energies. The reflectron is located at the end of the linear flight tube. It is composed of a series

of metallic rings and/ or grids with different voltages. The last electrode has voltage that is slightly higher than the ion initial acceleration voltage.

In the reflectron, ions enter the field free region and then penetrate into the reflectron. During the process, the energy of the ions is consumed. When all their energy has been lost, ions are reflected to the opposite direction of their motion and then finally arrive at the detector. The extent to which the ions enter into the reflectron depends on their energy content. The depth of penetration of ions into the reflectron is proportional to their kinetic energy content. In other words, the higher initial kinetic energy can be compensated by the longer flight path and higher acceleration out of the reflectron, consequently the overall flight times of the ions with same mass to charge ratio are made equal. The performance of the reflectron can be adjusted by varying the potential gradient along the series of the reflector. In case of linear potential gradient, it is a single-stage reflectron, and it focuses as the first order energy spread of ions. For further improvement, a dual stage reflector is applied to further increase the resolution.

The dual stage reflector is designed for second order energy focusing. It is composed of two stages where the field strength in the first stage is greater than the second one. This design enables the ions to lose two thirds of their kinetic energy in the first one-tenth of the reflectron depth. As these ions enter the second stage, they are retarded by weaker field strength. The lower potential gradient in second stage results in higher resolution. Because ions with similar energy can be separated with a longer path difference, this configuration can be finely adjusted to produce maximum resolution.

2.4 Instrumentation

The mass spectrometer used for this study is Bruker Biflex (Franzen, Germany). The instrument contains 5 basic components including laser system, ion source region, ion deflection, detection and reflector.

2.4.1 Laser System

The laser system provides a pulsed laser light at defined wavelength and intensity on a small target. The system consists of a pulsed UV N_2 laser with 337nm wavelength and 3ns pulse width. An attenuator allowed fine adjustment the laser fluence. The beam splitter is used to direct 5% of the laser light to a photodiode thus starting the time-of-flight measurement and triggering data acquisition by the digitizer. In addition, a lens system was installed to focus the laser beam and a mirror system to direct the beam into ion source on the target.

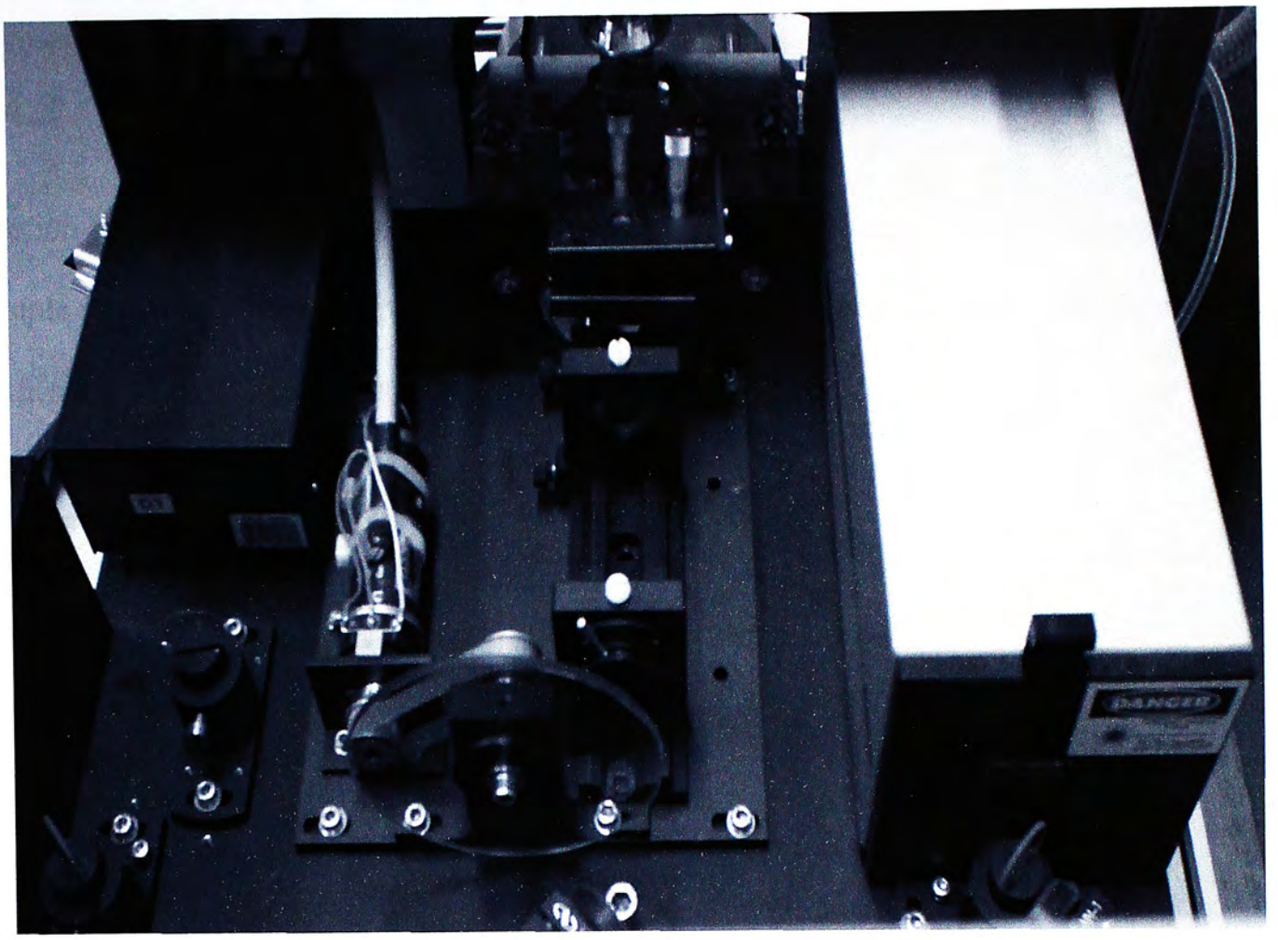


Figure 2.2 : A picture of laser and associated optics.

2.4.2 Ion source

The ion source consists of a positively or negatively charged metal electrode, i.e., the sample probe, an extraction plate and a ground-accelerating grid. Delayed extraction with a short delay time was used to increase the resolution of the instrument. In addition, the extraction voltage was set at 14.25kV and the lens voltage was 9.15kV to obtain the best performance. The instrument is evacuated to a pressure of 10^{-6} to 10^{-7} Torr by a turbo molecular pump (Balzer, Germany) backed by a rotary pump. Pressure inside the instrument is monitored by a flight-mounted ion gauge and the backing pressure by thermocouple gauge.

The scout ion source has an automated sample loading facility with automatic pump down and sample selection. Sample movement is fully computerized. Standard probes with 10 defined positions were used. A video CCD camera with monitor inside the SUN video display allows a better sample observation.

2.4.3 Ion Deflection

Ion deflection is used to deflect the undesired low mass ions, such as matrix or other irrelevant analyte ions. Deflecting this ion can improve the sensitivity of higher mass ions because detector saturation is avoided. The deflector is mounted behind the ion source and consists of two electrodes which apply 1.5kV/cm orthogonal to the ion beam for a short period of time. By the orthogonal field, ions are deflected away from the central optical axis thus cannot reach the detector.

2.4.4 Detection

The accelerated ions transit into the field free region, the flight tube, of approximately 1m. In the system, two detectors are used the linear detector and reflectron detector. In the linear detector, a microchannel plate detector (MCP) is used to collect the ions. In MCP detection, charged particles are incident at the input of a channel, then secondary electrons as well as further generations of electrons are generated. In our analysis, 1.5kV was applied across the microchannel plate.

2.4.5 Reflector

A dual stage gridless reflector mirror is applied. In the setup, ions are retarded in the first stage and then reflected by the second stage at +20kV. The reflector axis is inclined with respect to the axis of the ion beam to reflect the ion packets generated inside the ion source onto an off-axis electron multiplier. The gridless reflector design results higher ion transmission and resolution. In addition, the sensitivity is increased by focusing of the divergent ion beam due to the ion lens type design of the reflectron. In our experiments, reflector was applied and the detector was set to 1.7kV. An electron multiplier is used to detect the formed ions. Then, the ion signal is recorded by the digitizer.

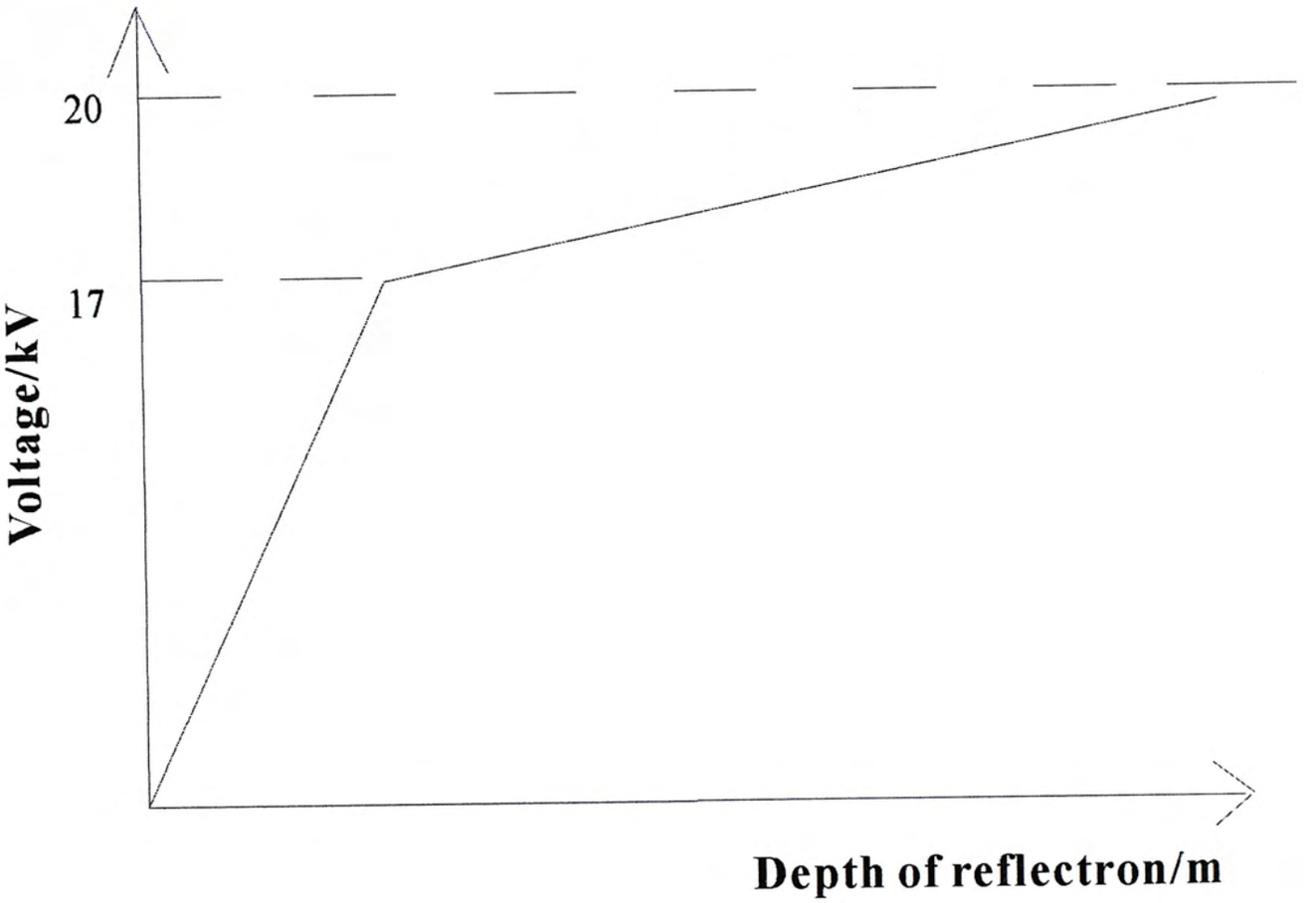


Figure 2.3 : Details of the reflectron voltage.

2.4.6 Data acquisition

Center to the data acquisition and computer control system is a microprocessor interfaced to an OS9 computer via a VME-bus. The OS9 computer is, in turn, controlled by the user through a SUN workstation (SPARC20) via ethernet. Settings for firing of laser, high voltages, sample target positions and the position of the gradient neutral density filter (i.e. the laser fluence) are all computer-controlled by the software XMASS.

Date acquisition is initiated by firing a laser pulse. A small portion of the laser photons is directed to a photodiode which in turn triggers a start signal for the transient digitizer (250MHz, LeCroy, US). Signals from the detector are pre-amplified, digitized and recorded by the digitizer. The digitized signals are then transferred to the SUN workstation for display and processing. The SUN workstation serves as a user-friendly terminal for instrument control, data acquisition and also as a powerful data processing device.

2.5 Experimental

2.5.1 Sample preparation

Dextran standards (1,000, 5,000 and 12,000) were purchased from Fluka Chemicals (Buchs, Switzerland). In sample handling, micro-scale auto-pipette fits with disposable tips were employed to deliver samples in volume 0.5 μl to 1.0 ml. In sample preparation, small PVC Eppendroff, maximum 1.5 ml, were used. In all

experiments, all the dextran sample, matrix and co-matrix were used without further purification. The solvent used was ultra-pure water (18 MΩ).

In conventional dry droplet method, samples were dried under ambient condition before loading into the mass spectrometer. In two-layered preparation method, the non-aqueous matrix preparation processes, the sample plate was heated to around 50 °C to accelerate the drying process and the aqueous sample and matrix layer was dried as the dry droplet method then.

2.5.1.1 Matrix preparation

Saturated aqueous matrices were prepared by dissolving excess amount of matrix materials in ultra pure water (18 MΩ). Excess matrix materials were settled by applying a microcentrifuge and the supernatant solution was used.

The aqueous matrix solution was prepared by mixing 9:1 (v:v) aqueous 2,5-dihydroxybenzoic acid (2,5-DHB) (20.0 g/L, Aldrich Milwaukee, Wisconsin, USA) and aqueous ammonium fluoride (NH_4F) (6.65 g/L, Aldrich Milwaukee, Wisconsin, USA). Different volume ratio of the co-matrix and 2,5-DHB were mixed to obtain different matrix/co-matrix ratio.

In the experiment of two-layered preparation, saturated 2,5-DHB was prepared by dissolving an excess amount of the matrix materials in pure acetone (AR grade, Labscan) and saturated NH_4F (Aldrich Milwaukee, Wisconsin, USA) was dissolved in pure methanol (HPLC grade, Labscan). Excess matrix materials were settled by applying a microcentrifuge. The non-aqueous matrix solution was prepared by

mixing 9:1 (v/v) 2,5-DHB solution (saturated in acetone) and NH_4F solution (saturated in methanol) then undergo five-fold dilution. It was noted that all of the matrix solutions were freshly prepared daily.

2.5.1.2 *Fractionation by GPC*

Dextran 5,000 was fractionated by gel permeation chromatography (GPC) with Sephadex G-50 superfine (Amersham Pharmacia Biotech, UK) using ultra-pure water (18 mΩ) as the eluent. The packing material was allowed to swell and equilibrate in ultra pure water and sonicated for 1 hour. A column with 60 cm x 1.6 (i.d.) cm was used. The flow rate was adjusted to 3 mL per hour and fractions were collected every hour. The elution process was monitored by testing the solution collected in each fraction against polysaccharide contents using standard ammonium molybdate/ H_2SO_4 treatment on a TLC plate. Molecular weight of each fraction was determined by MALDI-TOFMS and calibration curve was established by plotting the Ln (MW) against elution volume,

$$\text{Ln (MW)} = -0.0569 \text{ (Elution volume/ ml)} + 11.966$$

where Ln (MW) is the logarithm of the molecular weight of the fraction resulted.

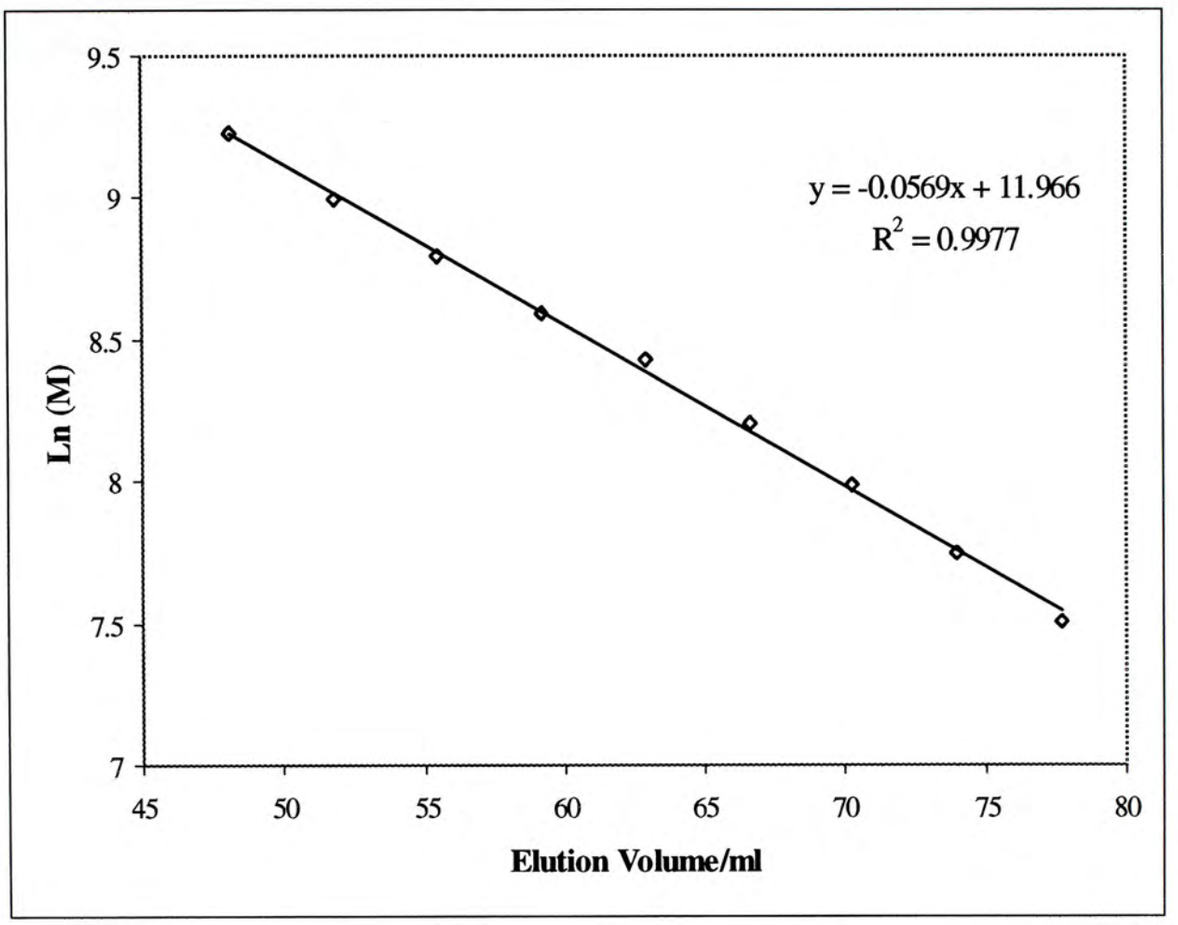


Figure 2. 4 Calibration curve of the Sephadex G-50 column.

Table 2. 1 Molecular weight information of various fractions of fractionated dextran 5,000 standard.

Fraction No.	1	2	3	4	5	6	7	8	9
$\mathbf{M_n}$	1,810	2,310	2,950	3,660	4,590	5,380	6,600	8,050	10,160

2.5.2 Calibration

Angiotensin II, ACTH, insulin and cytochrome C in 2,5-dihydroxybenzoic acid (2,5-DHB) were the calibrants to calibrate the TOF instrument externally. The error of the calibration is limited to 10 ppm.

2.6 Data analysis

Molecular weight of polymeric sample was defined by parameters, number average (M_n), weight average (M_w) and polydispersity (P.D.).

$$M_n = \Sigma n_i M_i / \Sigma n_i$$

$$M_w = \Sigma w_i M_i / \Sigma w_i$$

$$P.D. = M_w/M_n$$

In the equations, n_i is mole fraction and w_i is weight fraction. Typically, 100 shots were acquired for each spectrum and in order to improve the signal to noise ratio, it was processed by 10 point filtering. In the number average molecular weight (M_n), weight average molecular weight (M_w) and polydispersity (P.D.) calculation were performed in the Microsoft Excel by extracting the molecular weight and signal intensity information from XTOF.

CHAPTER THREE

USE OF AMMONIUM SALT AS CO-MATRIX

3.1 Introduction

In the last two decades, many important ionization techniques, such as electron impact ionization (EI), chemical ionization (CI), fast atom bombardment (FAB), thermospray ionization (TSI) and laser desorption (LD), have been developed for the analysis of organic compounds. The applications of these techniques were however limited by the low accessible mass range and the induction of extensive fragmentation of the molecular ions. The techniques of matrix-assisted laser desorption / ionization (MALDI) and electrospray ionization (ESI) provide much milder conditions for desorption and ionization of involatile and thermally labile molecules such as polymers and biomolecules. These techniques reduced the extent of molecular fragmentation during phase transition and ionization processes and extended the accessible mass range of the mass spectrometry to hundreds of thousand Daltons. Advantages of MALDI-MS and ESI-MS over other chromatography or biochemical methods are the simplicity, the high speed of analysis, and the accuracy of the analysis. Mass spectrometry provides both structural and molecular weight information for compounds of interest.

Apart from being a well-established technique for analysis of biological samples, MALDI-MS method has also successfully been applied to polar and non-polar synthetic polymer samples using different matrix systems.[74,75,76,36] For the analysis of narrowly distributed polymers, it has been demonstrated that molecular weight information obtained by MALDI is accurate and is consistent with that of GPC result. However, substantial discrepancy was found between MALDI and GPC

results for analysis of dispersed polymer. A systemic under estimation of the molecular weight information was found in the MALDI analysis. Many experimental parameters have been proposed to account for the observed mass discrimination in the analysis of polydispersed polymers by MALDI-TOF mass spectrometry. Examples include sample preparation methods, MALDI-induced fragmentation, mass dependent formation of multimers and multiply-charged ions, and the nature and concentration of cationization reagents[59,60,61,62,63]. Instrumental parameters affecting the ion transmission and ion-to-electron (or ion-to-ion) conversion[64,65] have also been found to limit the general application of MALDI-TOF technique to analysis of polydispersed polymers. Although factors affecting the MALDI analysis of dispersed polymers have previously been evaluated[56,57,58], the influence of the physical properties of the polymers has not been extensively determined. In particular, many polysaccharides are highly dispersed and have poor solubility in most solvents, including water.

In this Chapter, an attempt was made to evaluate the significance of molecular fragmentation on the MALDI analysis of dispersed polysaccharides. The possible use of co-matrices for alleviating MALDI-induced molecular fragmentation was also investigated. Finally, the so-called layer-by-layer sample preparation method was explored in an attempt to increase homogeneity of the sample prepared for MALDI analysis and to enhance spectral reproducibility.

3.2 Results and discussion

Figure 3.1(a-c) show the MALDI mass spectra of dextran standards, i.e. 1,000, 5,000 and 12,000 respectively, using 2,5-DHB as matrix. The samples were prepared using the conventional drop-drying method. In all spectra, the dextran molecules were detected in the form of sodiated and/or potassiated adduct ions. Two series of ion signals with mass increment of 162 Da were observed. This mass increment is consistent with the mass of the glucose repeat unit. The mass difference between the major and the minor ion series is 18 Da. In cases of dextran 5,000 and 12,000, the peak distributions were found to displace substantially towards the low-mass region and were highly asymmetric. Table 3.1 summarizes the molecular weight information obtained using MALDI-MS and the corresponding GPC values provided by the supplier. The molecular weight information measured using MALDI-MS method was consistently lower than that of GPC values. By mathematical transformation of the M_w information obtained using GPC into M_n information, the molecular weight distribution obtained by MALDI-MS can readily be compared with that of GPC. Figure 3.2 shows a comparison of the number-averaged molecular weight distribution obtained by MALDI-MS and GPC-RI methods for dextran 5,000. It was found that the difference between the values determined by MALDI-MS and GPC-RI increases as the average molecular weight of the polysaccharide increases. In order to identify the sources of the minor ion series and the significance of MALDI-induced fragmentation of dextran molecular ions, the fractionated portions

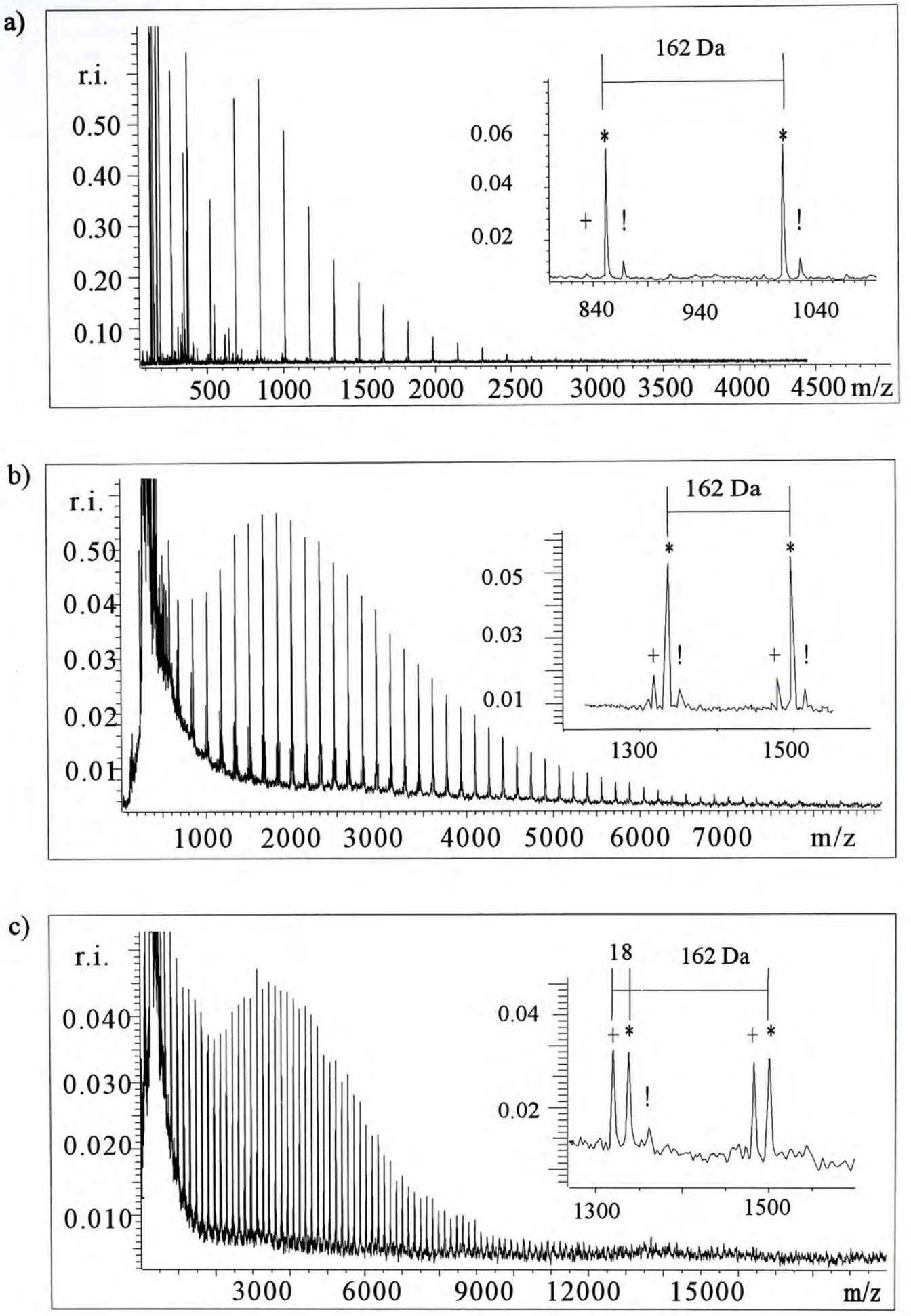


Figure 3.1 : Positive-ion mass spectra of (a) dextran 1,000, (b) dextran 5,000 and dextran 12,000 in 2,5-DHB matrix.

+ Fragment ion corresponding to the acid catalysed cleavage at glycosidic bond

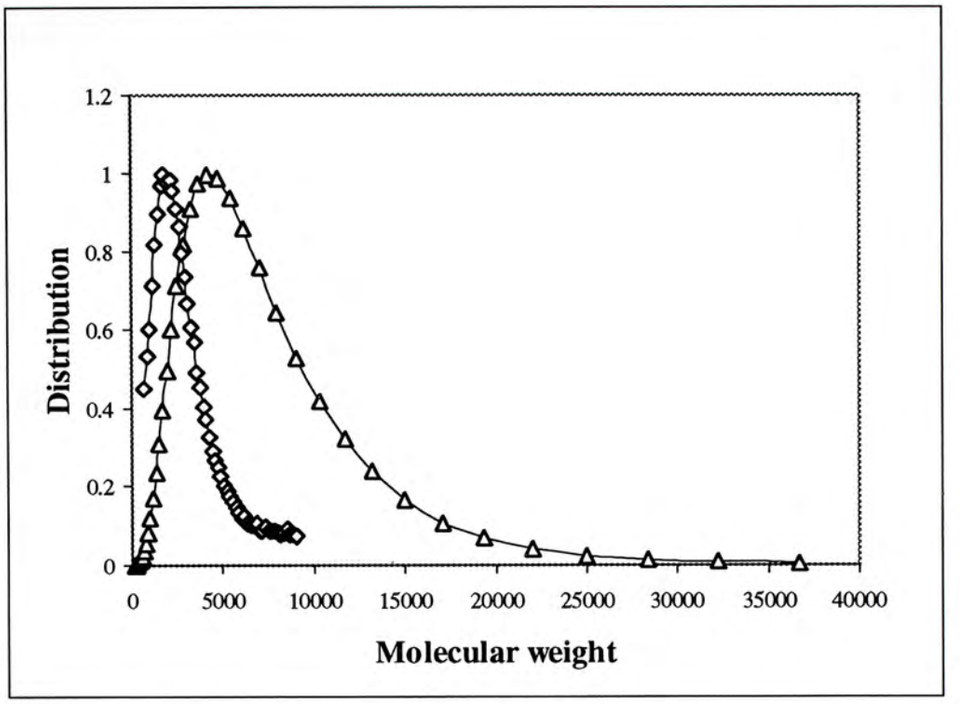
* Sodiated dextran molecular ion

! Potassiated dextran molecular ion

Table 3. 1 Summary of the molecular weight information obtained using MALDI-MS and the corresponding GPC values provided by the supplier.

		MALDI-MS	**GPC-RI**
	M_n	1,320	1,009
Dextran 1,000	M_w	1,540	1,271
	P.D.	1.2	1.3
	M_n	3,080	3,255
Dextran 5,000	M_w	4,260	5,221
	P.D.	1.4	1.6
	M_n	5,040	8,108
Dextran 12,000	M_w	7,590	1,1602
	P.D.	1.5	1.4

a.)



b.)

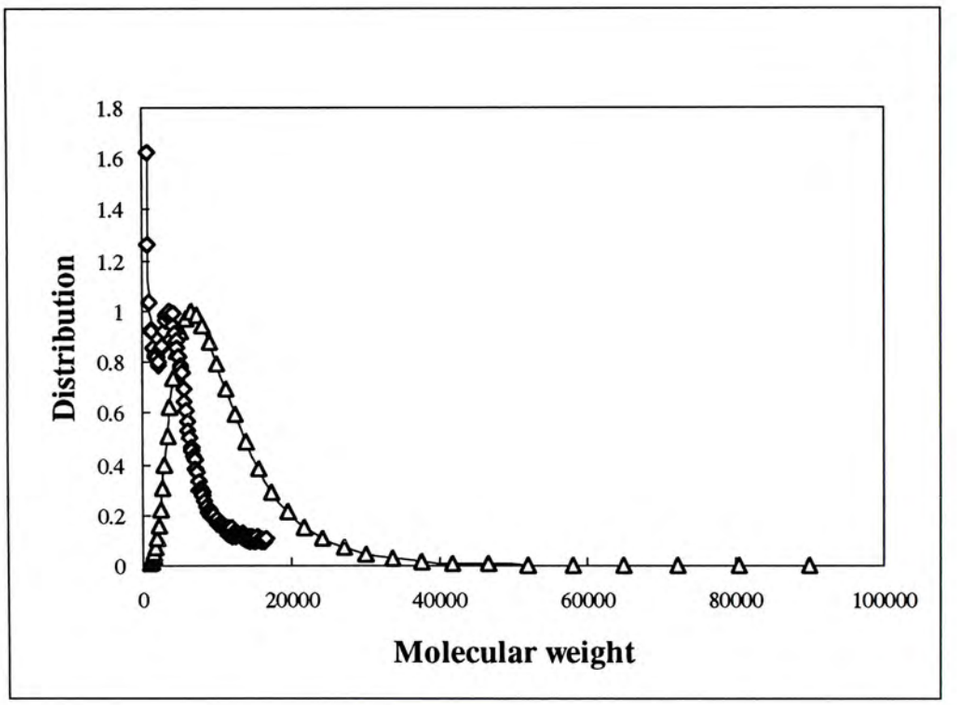


Figure 3. 2 A comparison of MWD of dextran 5,000 and 12,000 obtained from MALDI-MS (◇) with 2,5-DHB as matrix and GPC (△) provided by the supplier.

of dextran 5,000 were analyzed. Figure 3.3(a-c) show the positive-ion MALDI mass spectra of fractionated dextran samples. For samples with M_n < 3,000 Da, two symmetrically distributed ion series with a mass difference of 16 Da were observed. These ion series were tentatively attributed to the sodiated and potassiated molecule ions of dextran molecules. For samples with M_n > 3,000 Da, an additional low-mass ion series was observed. This ion series was found to be 18 Da lower than the presumably sodiated molecular ions of dextran. Since the dextran samples analyzed were fractionated from dextran 5,000 standard using GPC method, the low-mass ion series observed in Figure 3.2c must be fragmented from the high-mass dextran molecules during either the sample preparation and/or the desorption / ionization processes under MALDI conditions. Since high-mass dextran molecules tend to fragment more easily than low-mass components under MALDI conditions, the dextran fragmentation should therefore be associated with the desorption / ionization processes rather than the conditions used in the sample preparation. The glycosidic linkages in dextran molecules are formed from condensation of sucrose monomers with elimination of water molecules. This kind of (1→6) glycosidic linkage is relatively stable and can tolerate for moderate interval in acidic media at strength of pH 3.3 to pH 4.2 and at a temperature up to 120 °C. Extensive fragmentation will however result in highly acidic medium, i.e. pH < 3.0 at elevated temperature >> 120°C. Under typical MALDI sample preparation conditions for analysis of polysaccharides, i.e. 2,5-dihydroxybenzoic acid with a pH of 2 to 3, the acid induced cleavage of glycosidic linkage should not play a substantial role.

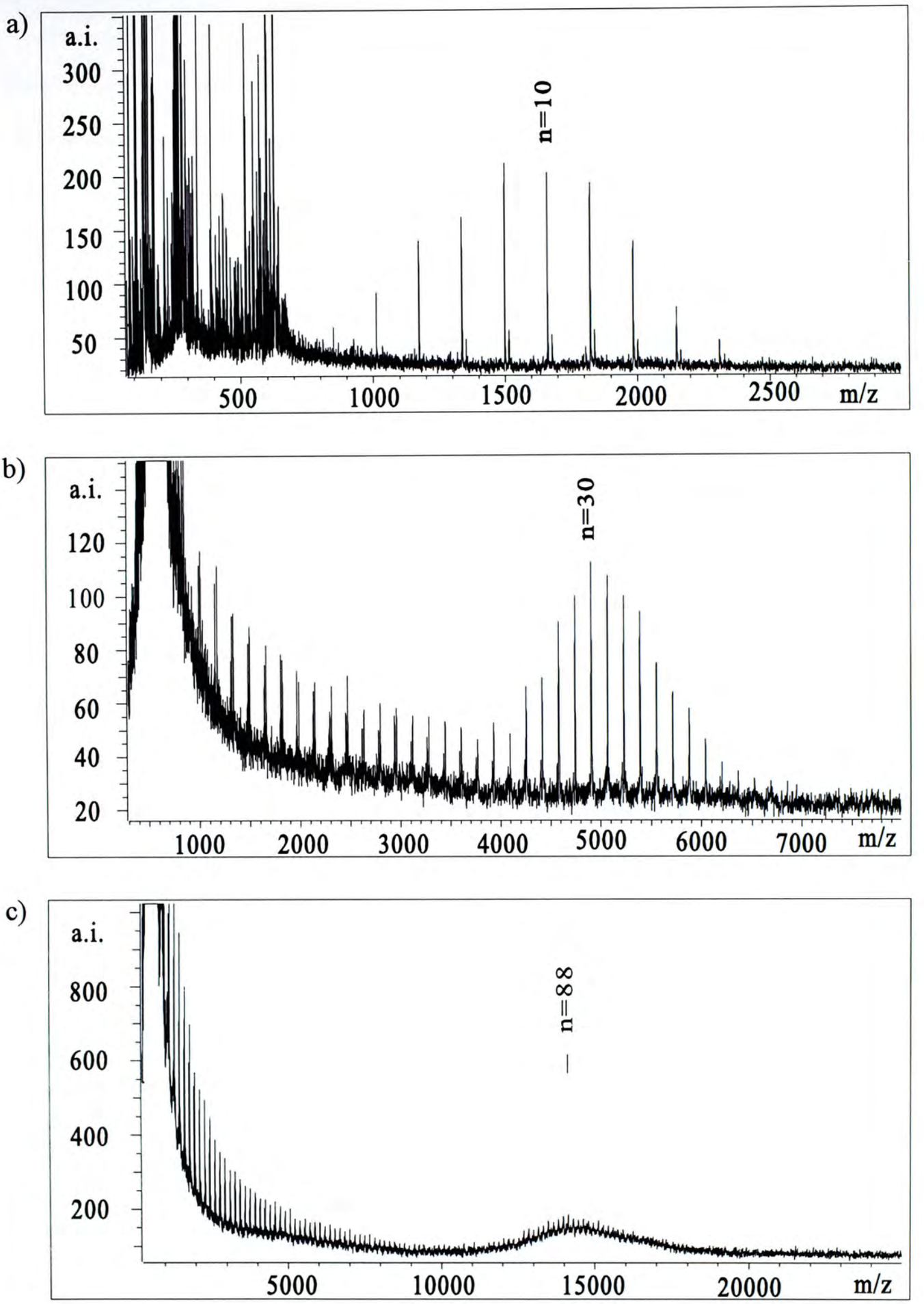
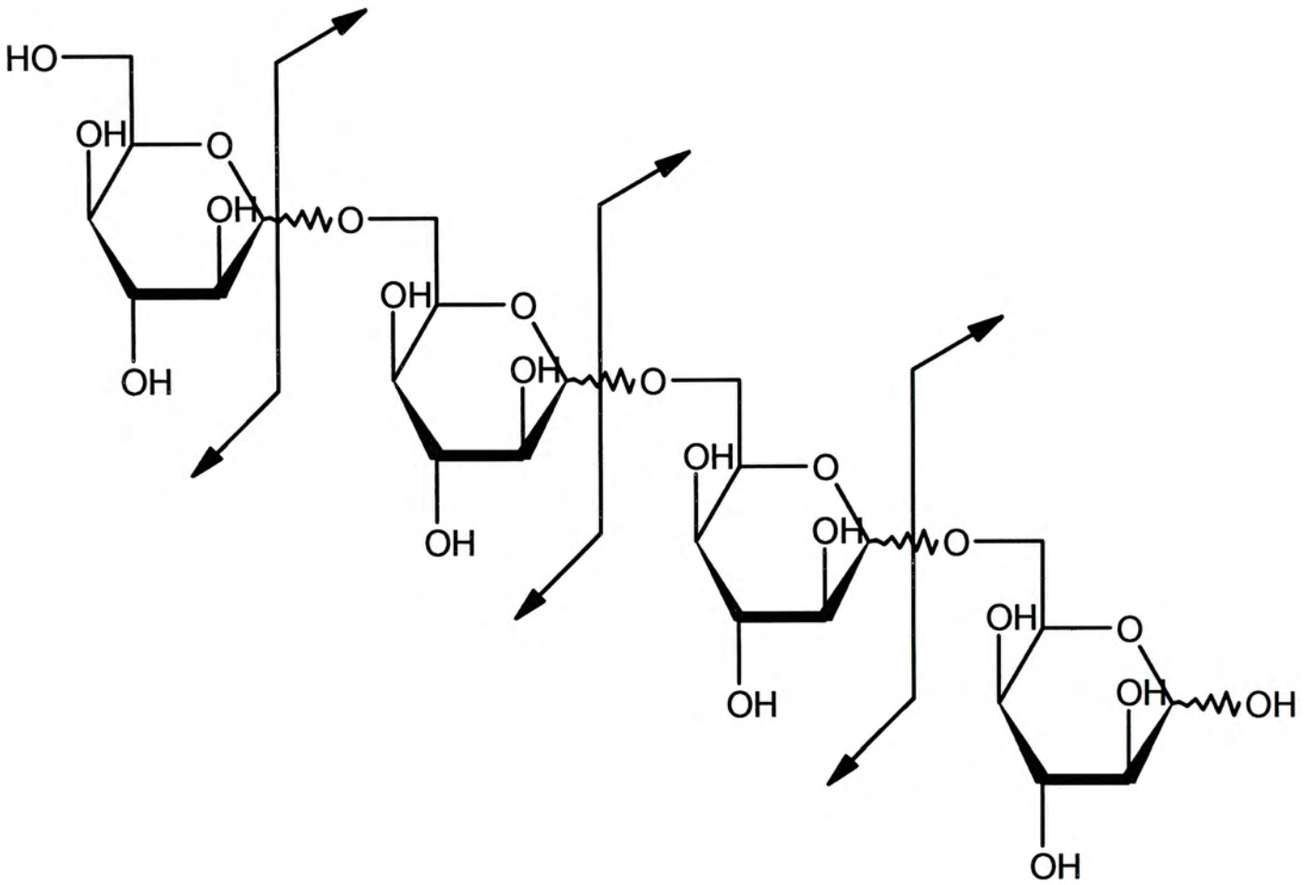


Figure 3.3:Positive-ion MALDI mass spectra of narrow dispersed dextran using pure 2,5-DHB as matrix, Mp at (a) n = 10; (b) n = 30; and (c) n = 88.

It is however postulated that the energy deposited by the laser photons might cause substantial elevation of sample temperature and lead to significant cleavage of glycosidic linkages during the desorption / ionization processes. This postulation is consistent with the fact that higher molecular weight polysaccharides require higher laser fluence for desorption / ionization and have stronger tendency for fragmentation. The fragmentation of carbohydrate was widely studied in Fast Atom Bombardment Mass Spectrometry (FAB-MS) and collision induced decomposition (CID) MS/MS.[77] It was noted that glycosidic bond was labile and extensive fragmentation would be resulted from low energy collision in CID (MS/MS). For alkali metal-cationized oligosaccharides, glycosidic fragments are consequence of C1-O cleavage with a transfer of a hydrogen to the oxygen by elimination across the proximal C1-C2 bond[78] and leads to different kinds of fragments from reducing and non-reducing as well as second glycosidic cleavage. The charge of the ion is provided by retention of the metal cation.

Figure 3. 4 Proposed glycosidic fragments of dextran.



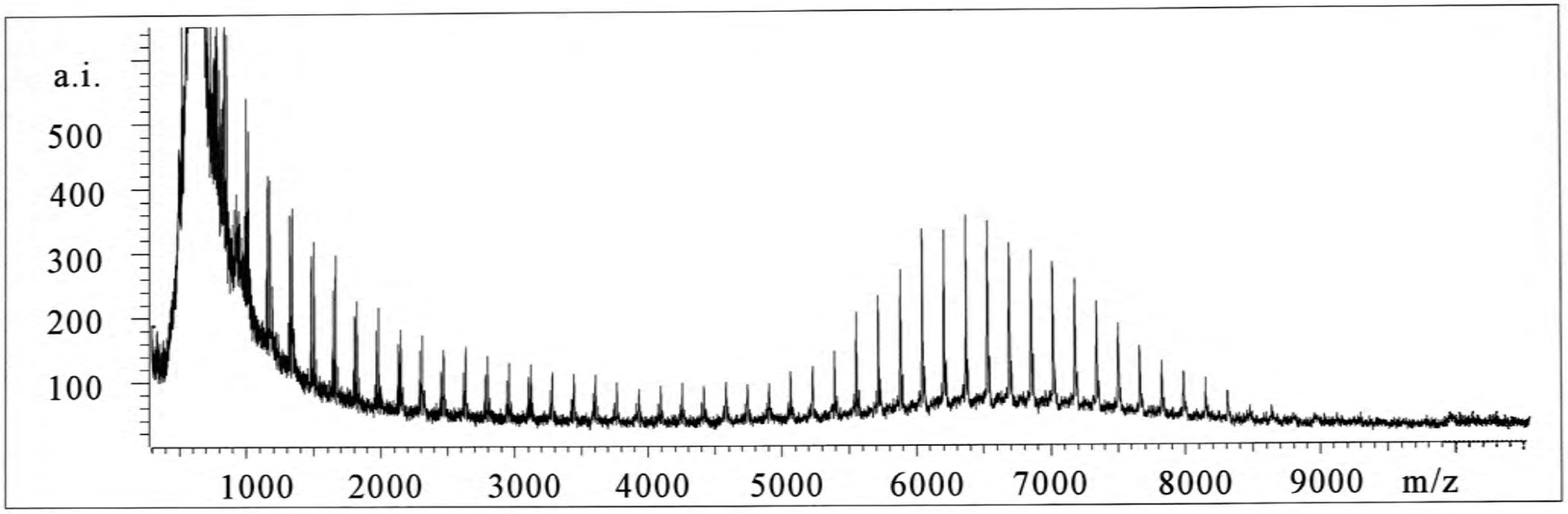
It is well-known that the extent of molecular fragmentation is matrix-dependent. Development of new matrix systems is thus a possibility in alleviating the fragmentation problem. Since the mechanism of action of matrix in the MALDI process is not well understood and the discovery of effective matrices is by-large trial-and-error, modification of the well-established matrix systems might provide an effective and promising method to improve their performances. Several substances, including fucose[79], 1-hydroxyisoquinoline (HIC)[80] and 2-hydroxy-5-methoxybenzoic acid,[81] have served co-matrices for 2,5-dihydroxybenzoic acid matrix to improve its analytical performance. Substantial improvements, in terms of resolution, sensitivity and reproducibility, have been observed in protein analysis. It was particularly noted that the extent of molecular fragmentation has also been suppressed using some of these co-matrices.

3.2.1 Effect of co-matrix

Since the fragmentation of dextran is believed to be catalyzed by the acidic nature of 2,5-DHB matrix at an elevated temperature during desorption/ ionization process(es), selection of co-matrix and additives substances were based on their ability to reduce the proton activity and/or the transition temperature of the desorption / ionization process(es). Two classes of co-matrices and additives were evaluated including basic organic substances and an ammonium salt with a high proton affinity anion. 3-Aminoquinoline (3-AQ) and 2-amino-5-nitropyridine (2,5-ANP) were two common basic matrices with absorption at 337nm. Ammonium fluoride was selected because of the high proton affinity of fluoride ions and the

superior performance of this material in assisting the MALDI analysis of DNA fragments.[82] Figure 3.5 shows the positive-ion MALDI mass spectra of fractionated dextran (fraction 7) using (a) neat 2,5-DHB; (b) 2,5-DHB/3AQ; (c) 2,5-DHB/2,5-ANP; and (d) 2,5-DHB/NH_4F matrices. In all cases, incorporation of these co-matrices and additive were found to reduce the degree of dextran fragmentation. For the working principle of the basic co-matrices, it is tentatively suggested that the increase in the pH of the resulting matrix system is responsible for the lowering of the extent of the acid-catalyzed cleavage of the glycosidic linkages. However, as the pH of the overall matrix system increases, their efficiencies in promoting desorption / ionization processes were found to be reduced substantially.[60] In addition, the spectral reproducibility of dextran using these basic co-matrices was found to be relatively poor. This was tentatively attributed to the mismatches between the solubility of 2,5-DHB and these basic co-matrices in water.

a)



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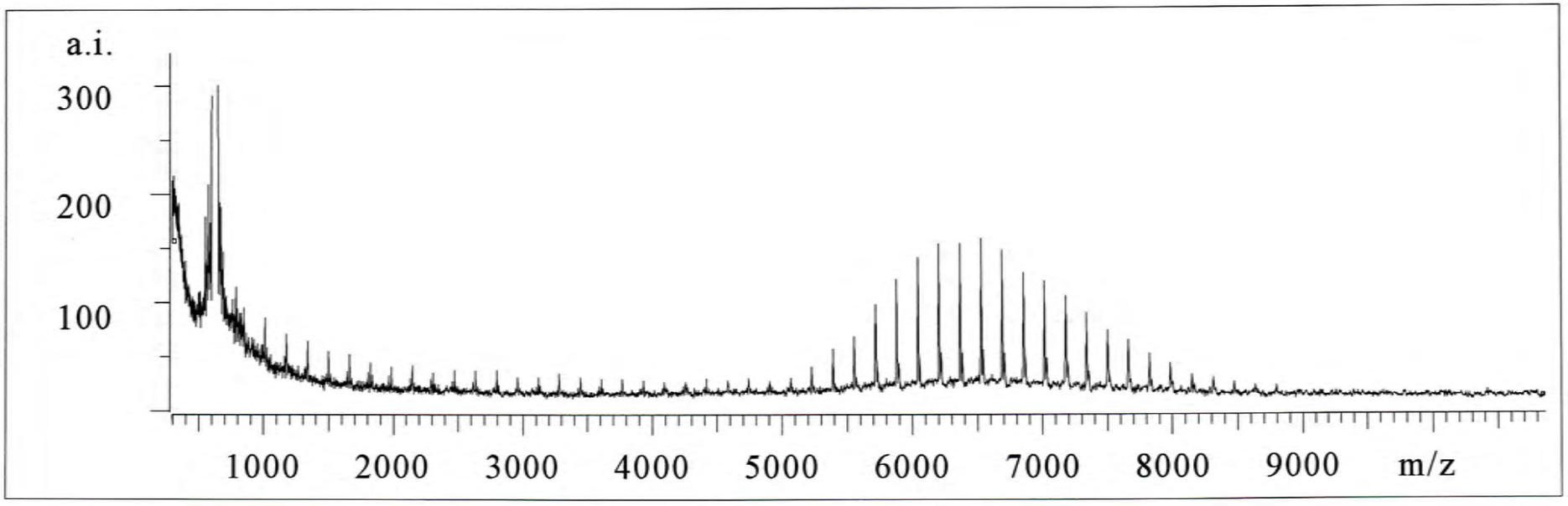
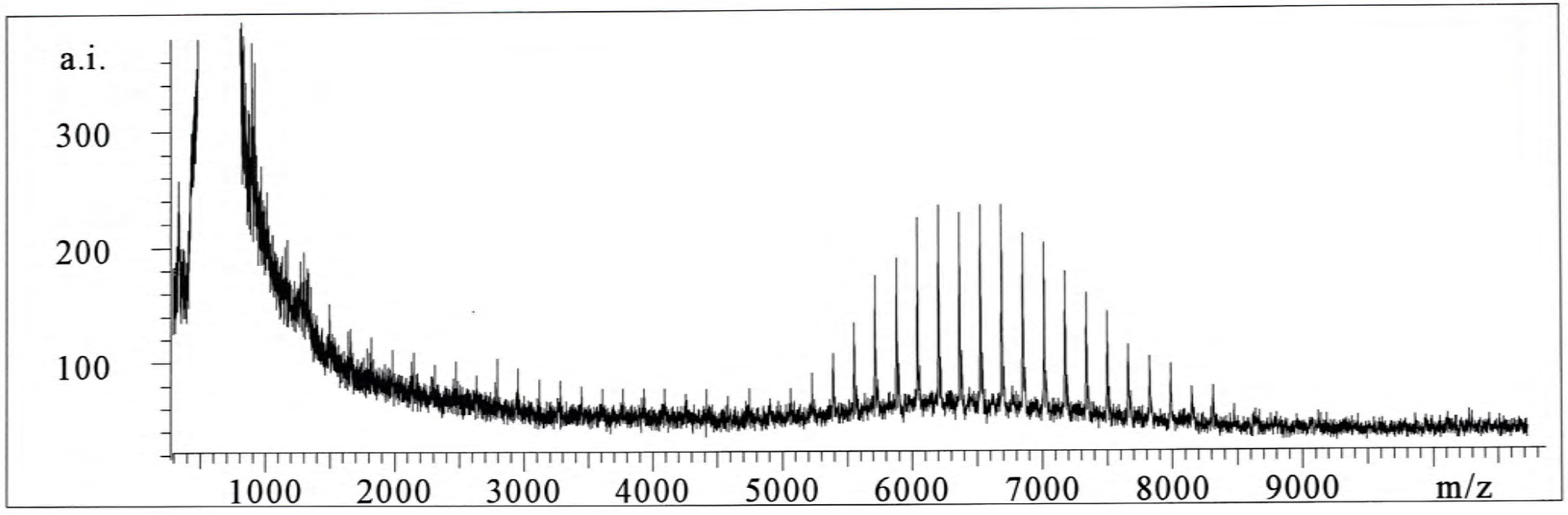


Figure 3.5 : Positive-ion MALDI mass spectra of fraction 7 using (a) 2,5-DHB, (b) 2,5-DHB/3AQ, (c) 2,5-DHB/2,5-ANP and (d) 2,5-DHB/NH4F matrices.

c)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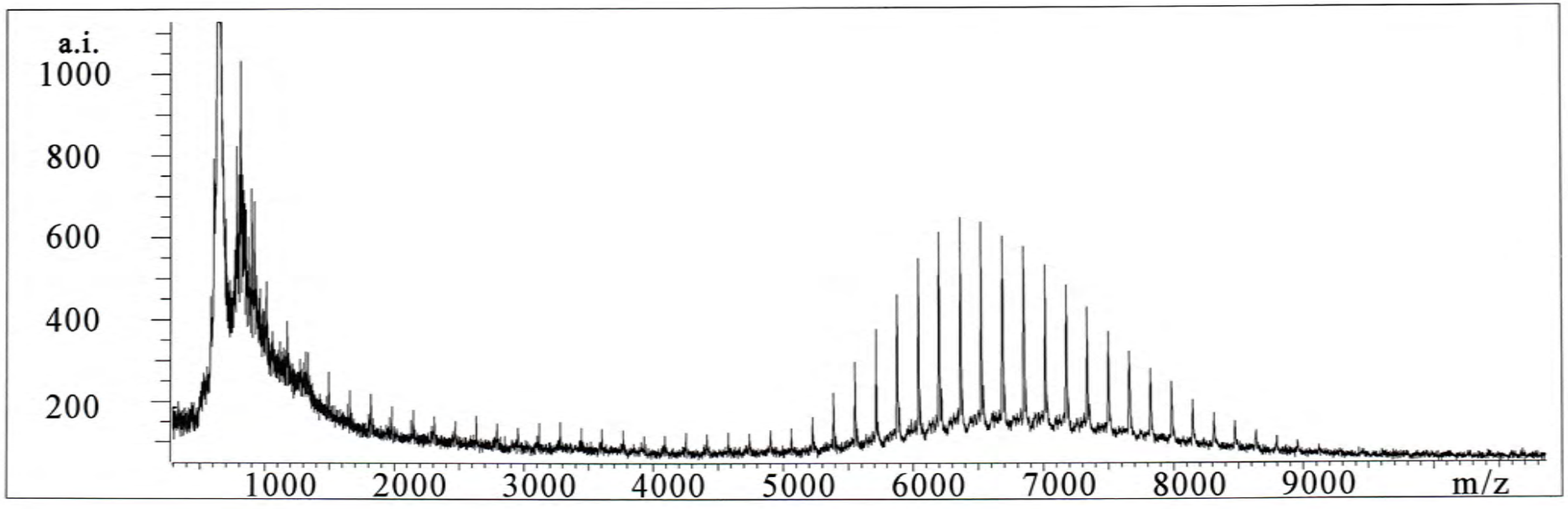
d)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The poor solubility of basic co-matrices might lead to partial segregation of the matrix and co-matrix components during the sample drying process. On the other hand, ammonium fluoride was found to reduce the degree of dextran fragmentation (see Figure 3.5d) without substantial reduction in the terms of sensitivity of analysis and spectral reproducibility. Although the exact mechanism of action remains largely unknown, it is believed that the fluoride anion might play a significant role in immobilizing excess protons in the sample, i.e. in the form of HF. In addition, a large number of ammonia molecules might be liberated from the ammonium ions upon laser irradiation. The frequent collisions among the cold ammonia molecules and the entrained dextran molecules might significantly lower the internal energy of the desorbed dextran molecules. Both the reduction of proton activity and the milder desorption / ionization conditions might therefore lead to a substantial reduction of the degree of dextran fragmentation. Using water-soluble ammonium fluoride as additive for 2,5-DHB matrix in the analysis of polysaccharides, a substantial visual improvement in the sample morphology was found. This improvement was also reflected from the ease of acquiring reproducible spectra without much effort in searching for "sweet-spots".

3.2.2 Effect of sample preparation

The sample preparation method has also been shown to exert substantial impact on the extent of sample decomposition in the MALDI process. Examples of sample preparation methods include dried-droplet method,[26] thin layer method,[83] fast[84]/ slow crystallization methods. One of the attributable reasons for preferential desorption of low-mass components for polydispersed sample might be associated with the

mismatch in the matrix-to-analyte ratios. It has been demonstrated that a higher matrix-to-analyte ratio was needed for analyzing high-mass analyte molecules. It is a general consensus that the amount of matrix per analyte molecule needs to be sufficiently high so as to preclude polymer entanglement and also to provide sufficient energy for phase transition in desorption/ ionization processes[56]. Li et. al. reported a 2-layered preparation method[85], mixture of analyte and matrix in aqueous solution was loaded on the top of the fast-evaporated matrix layer, resulting in higher reproducibility and sensitivity especially in the high mass molecules. In this study, the feasibility of applying this method on the analysis of dextran to increase the sensitivity of the high-mass fraction was also evaluated.

Figure 3.6a and 3.6b show the typical spectra of the dextran fraction 7 using drop-drying and 2-layer preparation methods, respectively. The introduction of the two-layer preparation method greatly improved the sensitivity of the MALDI mass spectra. The performance of the 2-layered system was more sophisticated, compared to the general drop-drying single-layer preparation. It was clearly shown that with the 2 - layered preparation method, the signal intensity was much higher and the reproducibility was also greatly improved. It was proposed that the improvement was caused by the formation of evenly distributed fine matrix crystals through rapid drying of high concentration of non-aqueous matrix solution at elevated temperature. This fine crystals were believed to provide nucleation sites for growth of analyte-matrix crystals.

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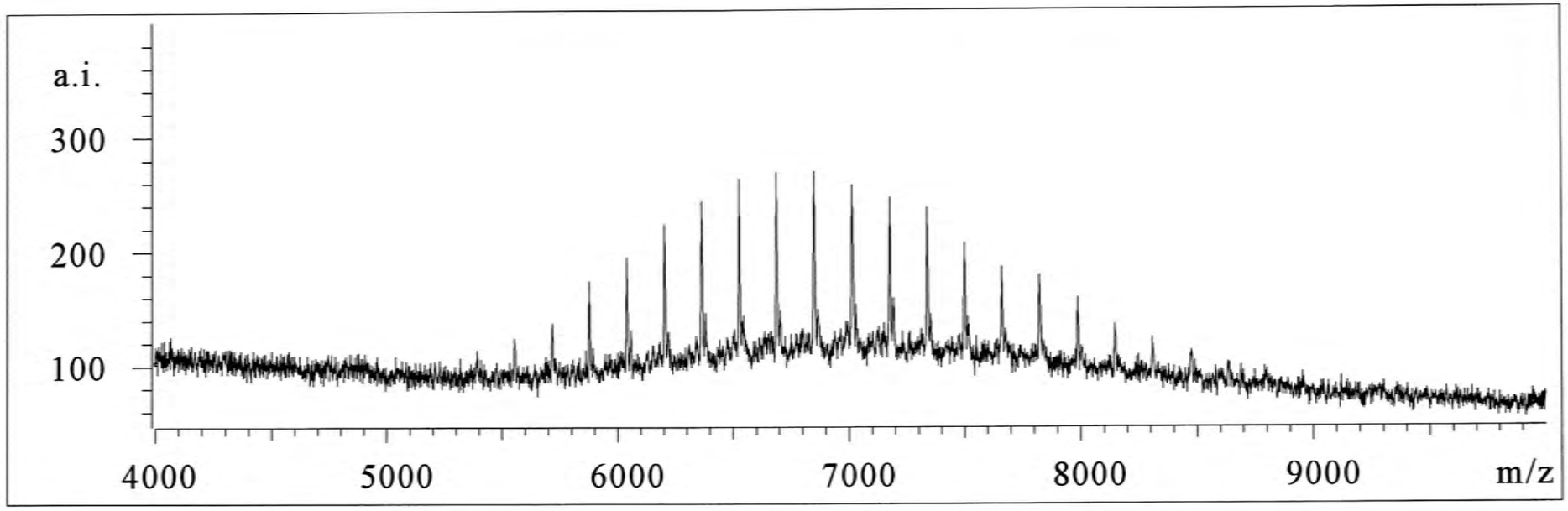
Figure 3.6a and 3.6b show the typical spectra of the dextran fraction 7 using drop-drying and 2-layer preparation methods, respectively. The introduction of the two-layer preparation method greatly improved the sensitivity of the MALDI mass spectra. The performance of the 2-layered system was more sophisticated, compared to the general drop-drying single-layer preparation. It was clearly shown that with the 2 - layered preparation method, the signal intensity was much higher and the reproducibility was also greatly improved. It was proposed that the improvement was caused by the formation of evenly distributed fine matrix crystals through rapid drying of high concentration of non-aqueous matrix solution at elevated temperature. This fine crystals were believed to provide nucleation sites for growth of analyte-matrix crystals.

a)



b)

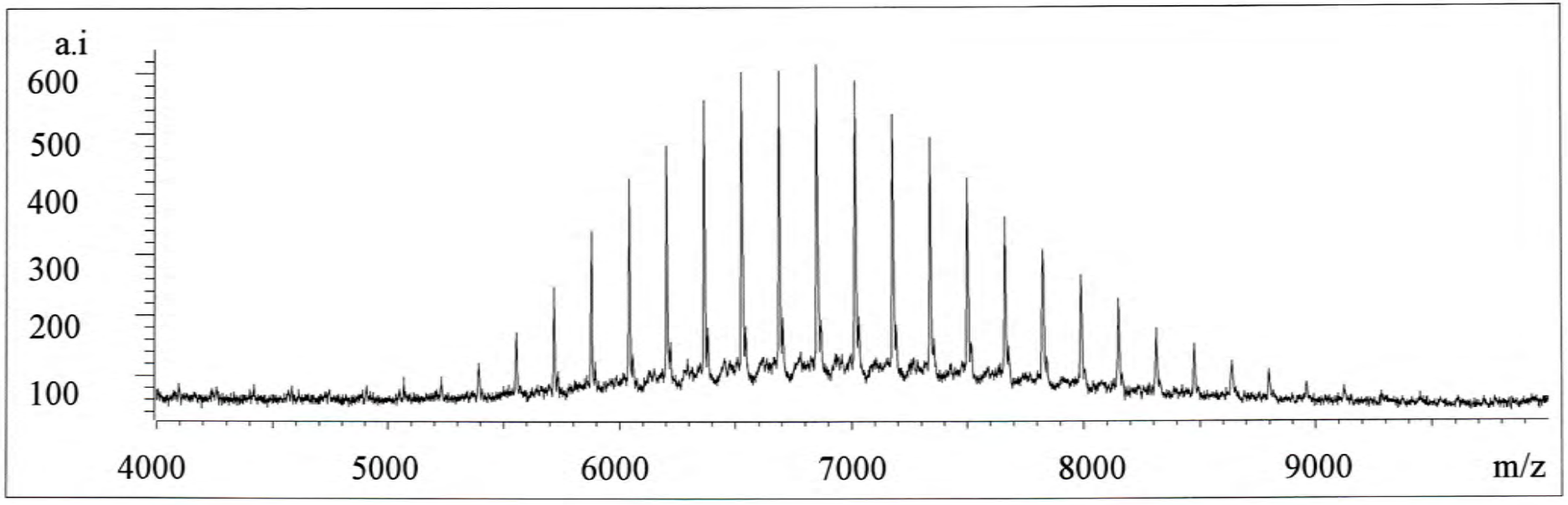


Figure 3.6 : Positive-ion MALDI mass spectra of fraction 7 using 2,5-DHB/ NH4F as matrix by a) dried-droplet method and b) two-layer preparation method.

3.2.3 Analysis of dispersed dextran

Figure 3.7 (a-c) show the spectra of dispersed dextran 1,000, 5,000 and 12,000 with 2,5-DHB/NH_4F matrix system using the 2-layered sample preparation method. The experimental molecular weight information is summarized in Table 3.2. In comparison with the results obtained using pure 2,5-DHB matrix and drop-drying sample preparation method (see Table 3.1), the molecular weight information determined in Table 3.2 are more consistent to that of GPC values. Much improvement was found for high-mass dextran 12,000. By alleviating the extent of molecular fragmentation during the MALDI processes, the contribution of fragment ion peaks at $(m-18)^+$ was removed from the spectra and hence from the estimation of the molecular weight information.

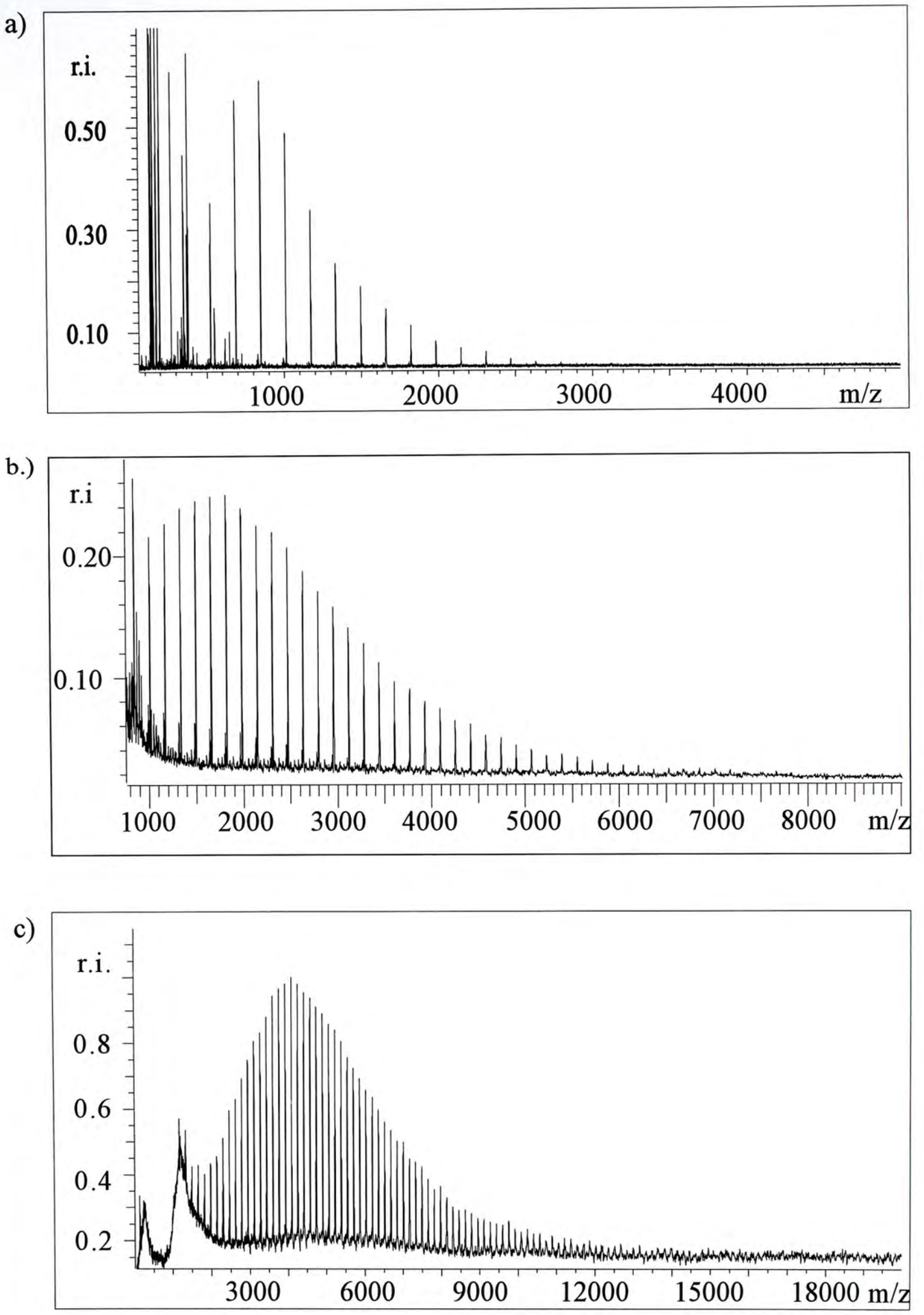


Figure 3.7 : Positive-ion MALDI mass spectra of dispersed (a) dextran 1,000, (b) dextran 5,000 and c) dextran 12,000 in 2,5-DHB/NH4F matrix

Table 3. 2 The molecular weight information measured by MALDI with modified matrix system and preparation method.

	Dextran 1,000	**Dextran 5,000**	**Dextran 12,000**
$\mathbf{M_n}$	1,290	3,690	6,780
$\mathbf{M_w}$	1,520	5,410	9,340
P.D.	1.2	1.5	1.4

3.3 Conclusion

This Chapter demonstrates the impact of fragmentation on the molecular weight measurement of dextran samples. A modified matrix system based on the use of ammonium fluoride co-matrix and a 2-layered sample preparation procedure were found to significantly alleviate the problems associated with fragmentation of dextran molecular ions during the MALDI processes. Substantial improvement in spectral reproducibility was also observed. Nevertheless, the molecular weight information obtained using these modified procedures remains different from that of GPC values. The discrepancies were still found to increase as the average molecular weight of the dispersed dextran increases.

CHAPTER FOUR

EFFECT OF SAMPLE PREPARATION

4.1 Introduction

The failure of MALDI method to analyze dispersed polymers is fundamentally interesting and deserves special attention. The molecular weight distribution measured under MALDI conditions was significantly displaced towards the low-mass region and was highly asymmetric[28]. Many experimental parameters have been used to account for the observed mass discrimination in the analysis of polydisperse polymers by MALDI-TOF mass spectrometry. Although factors affecting the MALDI analysis of dispersed polymers have previously been evaluated,[86, 87] the influence of the physical properties of the polymers has not been determined. In particular, polysaccharides are highly dispersed and have poor solubility in most solvents, including water. In this chapter, an attempt has been made to investigate the possibility and significance of generating a molecular weight gradient alcross the thickness of the sample during the sample crystallization. Such on-probe fractionation might become significant when the high-mass component reaches its precipitation point before the low-mass component and is not included in the matrix efficiently.

4.2 Experimental

4.2.1 Sample preparation

Two types of matrix solution, namely aqueous and non-aqueous, were prepared.

aqueous matrix solution was used for experiments involving conventional drop drying[65] and electrospray deposition methods[88]. The non-aqueous matrix solution was used for experiments involving a layer-by-layer sample preparation method. Mixtures of different mass fractions were prepared and analyzed by using four different sample preparation methods. Two of them utilized the "two layer method".[85] In this method, a matrix-only layer was first prepared by drying 0.5 μL non-aqueous matrix solution onto a sample plate. Matrix-mixed mass fractions were prepared by mixing 0.5 μL of selected dextran fractions and 32 μL aqueous matrix solution. The high-mass matrix-mixed fraction was loaded on the top of the matrix-only layer. A second matrix-only layer was prepared by applying and drying another 0.5 μL non-aqueous matrix solution. The low-mass matrix-mixed fraction was then added on the top of the second matrix-only layer. In the second layer-by-layer sample preparation method, the sequence of application of the matrix-mixed dextran fractions was reversed, i.e. the high-mass matrix-mixed fraction was loaded after the low-mass matrix-mixed fraction.

The other two methods involved the use of solution mixture of the high-mass and the low-mass fractions. The solution mixture was prepared by mixing equal volume of low-mass and high mass dextran fractions (0.5 μL each) and the aqueous matrix solution (64.0μL). One method employed the conventional drop drying method whereas the other utilized a pneumatic-assisted electrospray deposition method. In the drop-drying method, the solution mixture was dried under ambient conditions.

In the pneumatic-assisted electrospray deposition, a commercially available

electrospray source (Analytical Bradford, CT) was modified to include a stage to fix the sample plate. The solution mixture was pumped at a flow rate of 1 μL/hr and a nebuilizing gas (nitrogen) was adjusted to flow under a constant pressure of 0.5 psi to assist the removal of excess solvent.

All sugar containing fractions were freeze-dried and were dissolved in equal volume of ultrapure water (500 μL) to preserve their original relative solution concentrations

4.3 Results and discussion

As shown in the last chapter, in dispersed polymer analysis the peak distributions were found to displace substantially towards the low-mass region and were highly asymmetric. The number-averaged molecular weights of these dextran samples were found to differ significantly from the values obtained using gel permeation chromatography. These discrepancies were found to increase as the average molecular weight of the polysaccharide increases. In order to evaluate the possibility and the significance of the on-probe fractionation across the thickness of the sample crystals, four types of sample preparation methods have been designed to analyze different mixtures of high-mass and low-mass narrow distributed dextran samples. The mass of the polysaccharide used in this investigation was relatively low to avoid any complications introduced by detector saturation and the formation of multimers. In the present case, a dispersed dextran 5,000 was selected and was fractionated to produce several narrow distributed dextran samples with M_n ranging from 2,200 Da

to 12,000 Da. The polydispersities of these fractionated dextrans were determined to be less than 1.10. The molecular information obtained for fractions 19 to 24 are summarized in Table 4.1. The sample loading for MALDI analysis was carefully controlled to minimize sample aggregation. The amount of sample loading was kept to the minimum quantity required for producing a reasonable intensity (or signal-to-noise ratio) for the high-mass fraction, i.e. fraction 19 with M_n ~ 12,000 Da.

In a typical MALDI analysis, drop-drying method was applied. In the case, a small volume of solution mixture of the analyte and the matrix materials is applied onto a suitable substrate, such as stainless steel, and is allowed to dry under ambient air or controlled environment. During sample drying, the solvent evaporates gradually; and the analyte and the matrix crystallize when they reach their saturation points. For a mixture of analytes, their time-frames of crystallization depend critically on their relative solubilities in the solvent used. Since the solubility of a mixture of homologous, such as polymers, is always as a function of the molecular weight, the analyte crystallization is expected to start from the highest molecular weight components and shift gradually to the lower mass components.

Table 4. 1 Molecular weight information of the dextran fractions determined by MALDI-TOFMS

Fraction No.	24	23	22	21	20	19
M_n/ Da	2,130	5,350	6,340	7,700	9,660	12,140
M_p/ Da	2,126	5,207	6,342	7,638	9,420	11,530

The significance of the molecular weight gradient across the thickness of the sample deposit is therefore expected to relate closely with the range of the molecular weight of the polymer sample, i.e. the polydispersity (P.D.). This is particularly true if the solubility of the polymer in the selected solvent is not good.

Figure 4.1(a), 4.1(b) and 4.1(c) show the positive-ion MALDI mass spectra of fraction 24 mixed with fraction 23, 21 and 19, respectively, using the conventional drop drying method. Using the peak average molecular weight (M_p) as an index, it is possible to obtain the net intensity ratio between the low-mass fraction and the high-mass fraction for different dextran mixtures. As expected that the intensity ratios were found to increase as the mass ratio (M_n of the high-mass fraction / M_n of the low-mass fraction) increases. Table 4.2 summarizes the result obtained. The increasing intensity ratio for dextran mixtures with higher mass ratios can be attributed to the increasing polydispersity values (i.e. on-probe fractionation) and/or to the relative abundance of different oligomeric species (since the relative concentrations of various fractions were preserved after column fractionation).

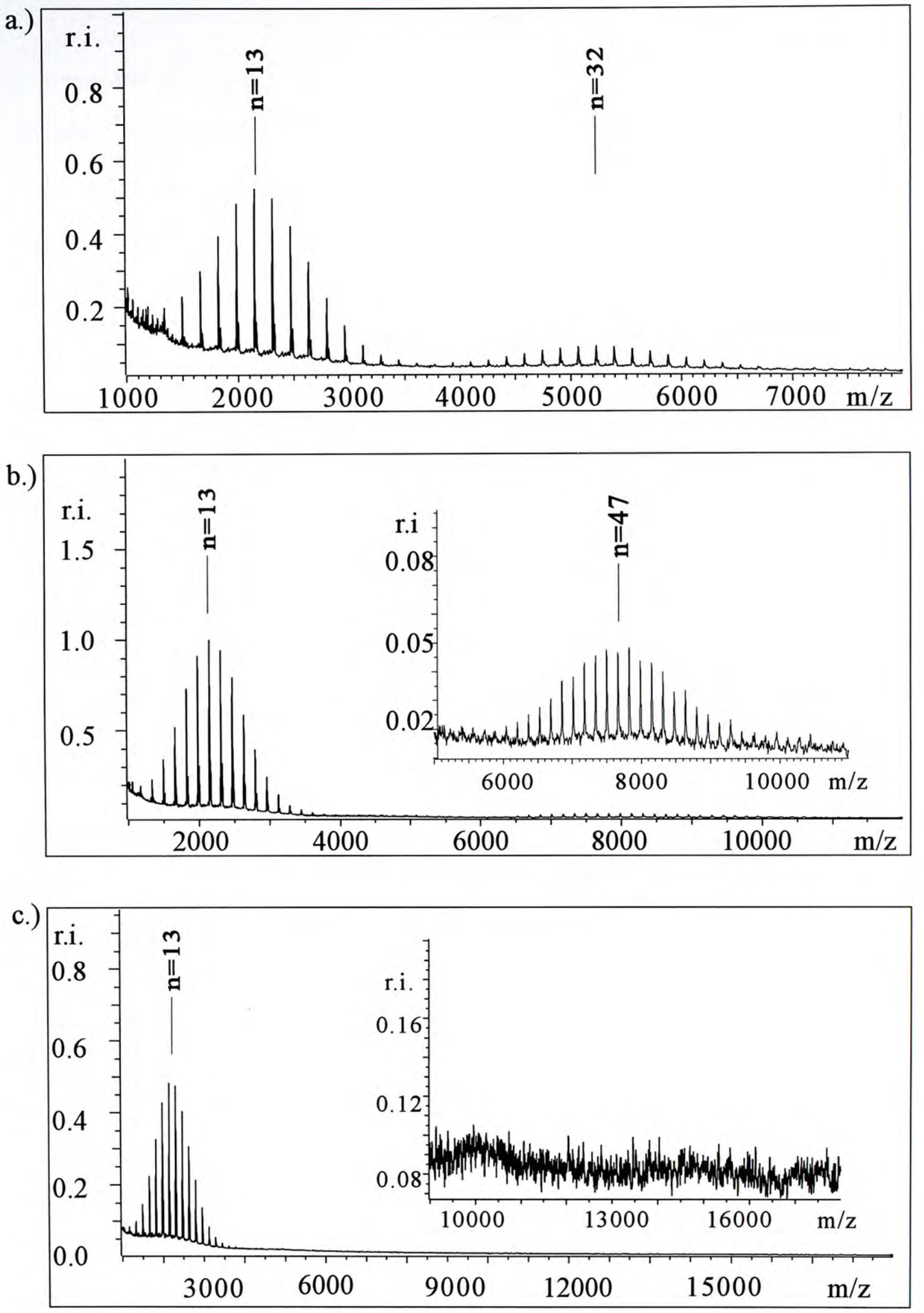


Figure 4.1 : Positive-ion MALDI mass spectra of fraction 24 mixed with fraction (a) 23, (b) 21 and (c) 19 respectively, using conventional drop drying method. 2,5-DHB was used as matrix.

In order to evaluate the importance of the on-probe fractionation, the same experiments were repeated by using layer-by-layer methods. Instead of mixing the high-mass and low-mass fractions in solution, they were successively applied and dried onto the sample probe. To minimize mixing between these dextran fractions, a layer of matrix-only crystals was applied on top of the first dextran layer and prior to the application of the second dextran fraction. If no re-dissolution of the dextran molecules from the lower layer occurs, this sample preparation method should only yield ions corresponding to the dextran fraction on the upper layer. Figure 4.2(a), 4.2(b) and 4.2(c) show the positive-ion MALDI mass spectra of fraction 24 mixed with fraction 23, 21 and 19, respectively, using the layer-by-layer method with the high-mass fractions (i.e. fraction number 23, 21 and 19) being placed on the upper layer. It is evidenced that substantial re-dissolution of the molecules from the lower dextran layer occurs during the sample preparation. In Figure 4.2(a-c), low-mass dextran remains the dominant analyte signals. However, in comparison to Figure 4.1(a-c), substantial enhancement of the signal corresponding to the high-mass dextran are however observed. Figure 4.3(a), 4.3(b) and 4.3(c) show the positive-ion MALDI mass spectra of fraction 24 mixed with fraction 23, 21 and 19, respectively, using the layer-by-layer method with the low-mass fractions (i.e. fraction number 24) being placed on the upper layer. No signal corresponding to the fraction 20 and 19 can be found in the samples prepared by using mixture of fraction 24 and 19 and mixture of fraction 24 and 20, respectively. All intensity ratios are summarized in Table 2. Figure 4.4 plots the intensity ratios of the low-mass component and the high-mass component as a function of the mass ratios. Similar to the drop drying

method, samples prepared by using layer-by-layer methods produced intensity ratios that increase as the mass ratio of the dextran fractions increases. However, it is interesting to note that the net intensity ratios obtained under drop drying method using samples of relatively low mass ratios (e.g. mixture of 24 & 23; 24 & 22) resemble more to the layer-by-layer method in which the high-mass fraction lies over the low-mass fraction. As the mass ratio increases, the net intensity ratios obtained under drop drying method (e.g. mixture of 24 & 20; 24 & 19) resemble more to the layer-by-layer method in which the low-mass fraction lies over the high-mass fraction. This observation is therefore taken as an evident to show the existence of on-probe fractionation. As the mass ratio increases, re-dissolution and re-arrangement of the low-mass components to the surface of the crystal becomes more significant and thus leading to a much higher enhancement of the signal intensity of the low-mass component (or suppression of the signal intensity of the high-mass component).

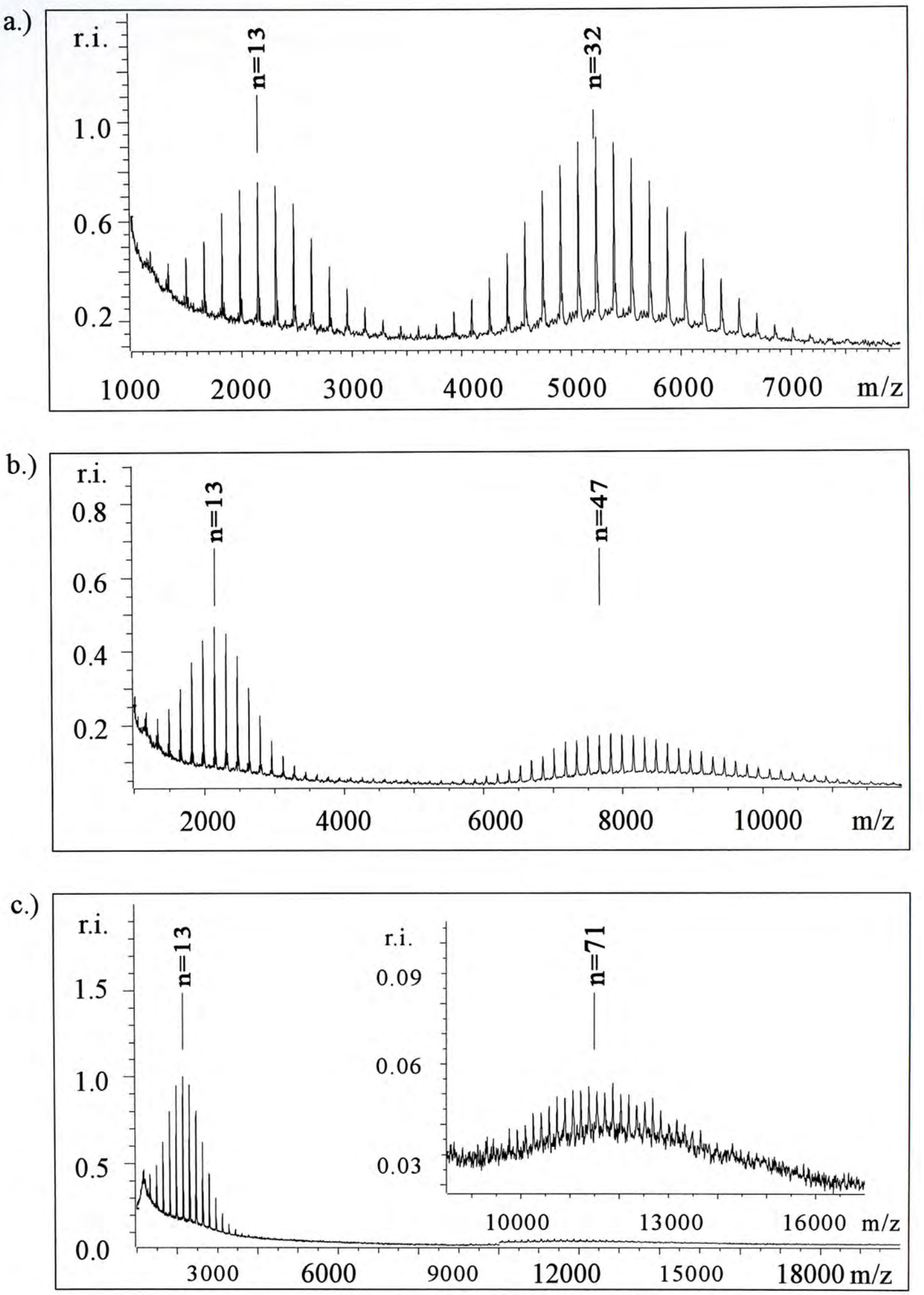


Figure 4.2: Positive-ion MALDI mass spectra of fraction 24 mixed with fraction (a) 23, (b) 24 and (c) 19 respectively, using the layer-by-layer method with the low-mass fraction (i.e. fraction 24) being placed on the lower layer. 2,5-DHB was used as matrix.

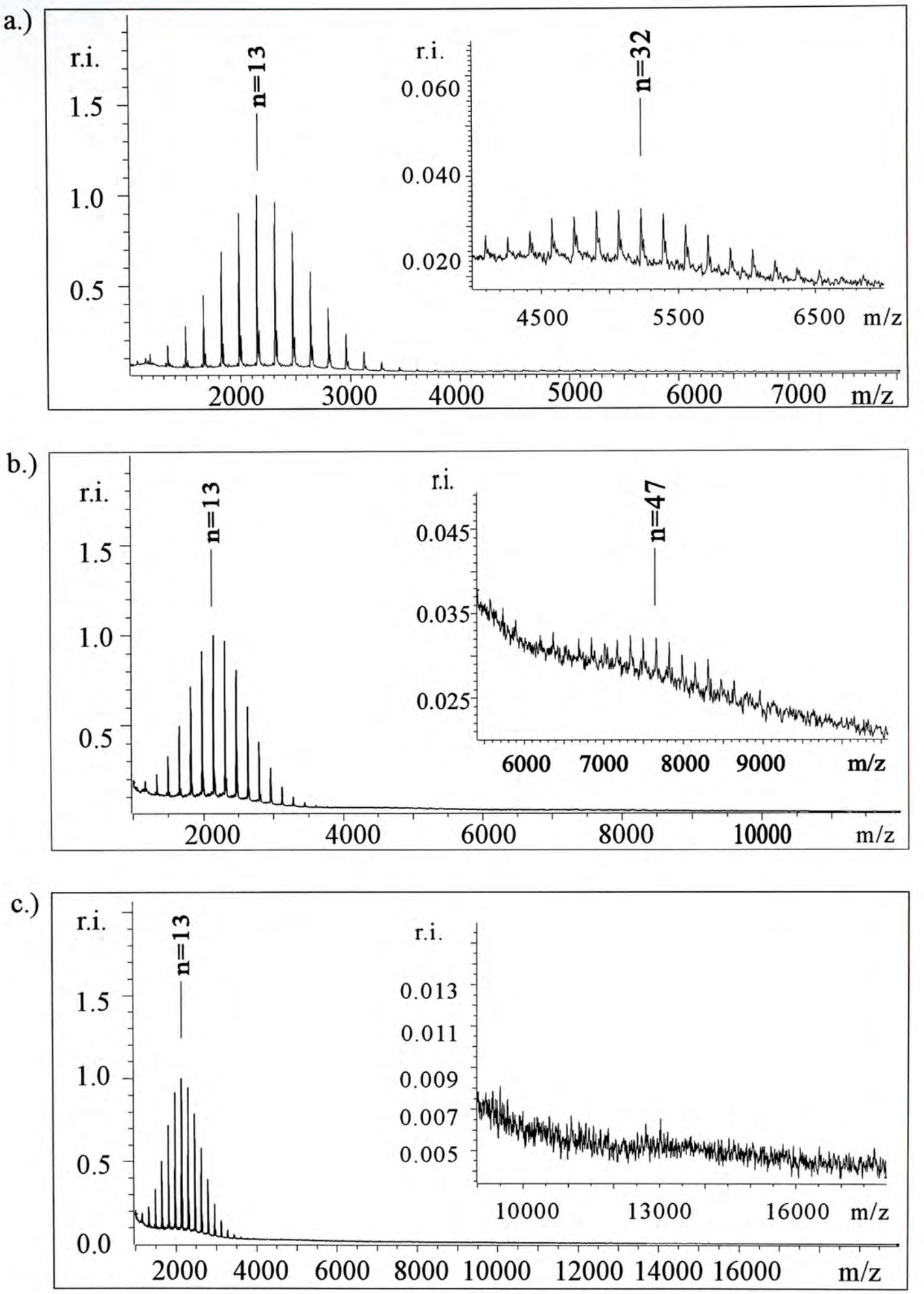


Figure 4.3 : Positive-ion MALDI mass spectra of fraction 24 mixed with fraction (a) 23, (b) 24 and (c) 19 respectively, using the layer-by-layer method with the low-mass fraction (i.e. fraction 24) being placed on the upper layer. 2,5-DHB was used as matrix.

In order to verify our postulation, samples prepared for drop drying experiments were analyzed again by using electrospray deposition technique. In this set of experiments, the dextran mixtures were sprayed using a conventional electrospray ion source. In attempt to avoid any possible re-dissolution and rearrangement of dextran molecules after deposition, the spray was pneumatically assisted with a flow of dry nitrogen. The sample plate was kept dry visually during the spraying process. The result is showed in figure 4.4. The results are summarized in Table 4.2 and are plotted in Figure 4.5. Using dextran fractions of low mass ratios, the samples prepared by electrospray deposition resemble that of the drop drying method. As the mass ratio increases, significant deviation of the net intensity ratio was found between these two methods. Electrospray deposition produces a much lower intensity ratio for samples of high mass ratios. These results reveal that on-probe fractionation becomes significant when the mass ratio of the two components increases. This is especially important if the sample is allowed to dry slowly.

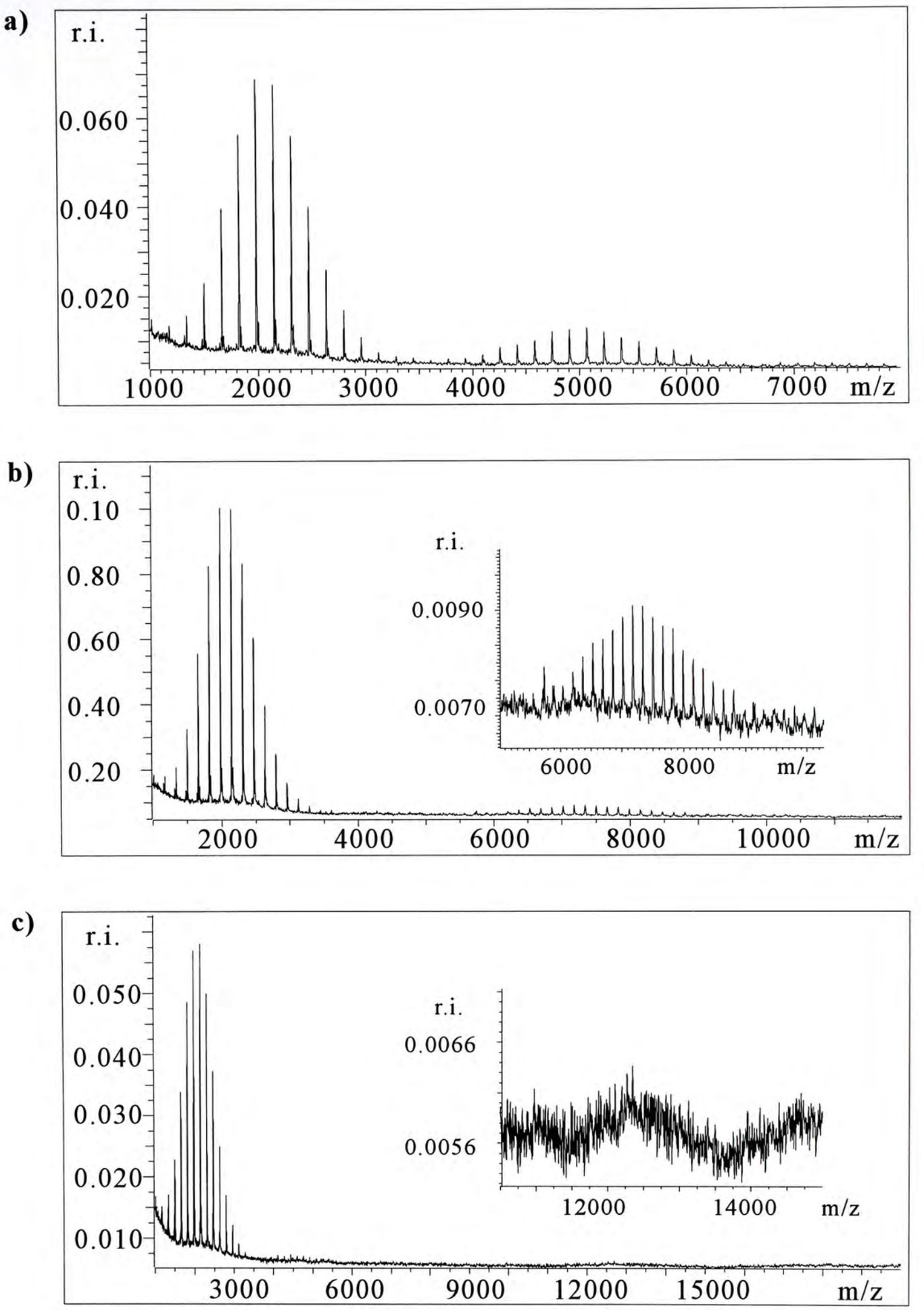


Figure 4.4 : Positive-ion MALDI mass spectra of fraction 24 mixed with fraction (a) 23, (b) 24 and (c) 19 respectively, using the electrospray method . 2,5-DHB was used as matrix.

Table 4. 2 The net intensity ratio of low-/high-mass fractions under different sample preparation methods

		Net Intensity ratio of Low-mass/High-mass fraction[b]			
Fraction No.	**Mass ratio [a]**	**Layer-by-layer deposition (low-mass over high-mass)**	**Drop-dry deposition**	**Layer-by-layer deposition (high-mass over low-mass)**	**Electrospray deposition**
24 and 19	5.4	Infinite	Infinite	65.4 (17.6)	Infinite
24 and 20	4.6	Infinite	163.3 (23.0)	11.8 (2.9)	77.3 (4.4)
24 and 21	3.8	202.1 (54.9)[c]	56.7 (7.3)	3.4 (0.5)	46.8 (4.0)
24 and 22	2.9	148.1 (18.2)	18.9 (0.5)	1.3 (0.1)	15.0 (1.0)
24 and 23	2.4	88.0 (19.0)	8.0 (0.3)	0.8 (0.1)	10.5 (1.3)

[a] Dividing the M_p of the high-mass fraction by the M_p of the low-mass fraction

[b] Dividing the intensity of low-mass fraction at M_p by that of the high-mass fraction at M_p. Signals were noise subtracted

[c] The value in bracket is the standard deviation from three replicates.

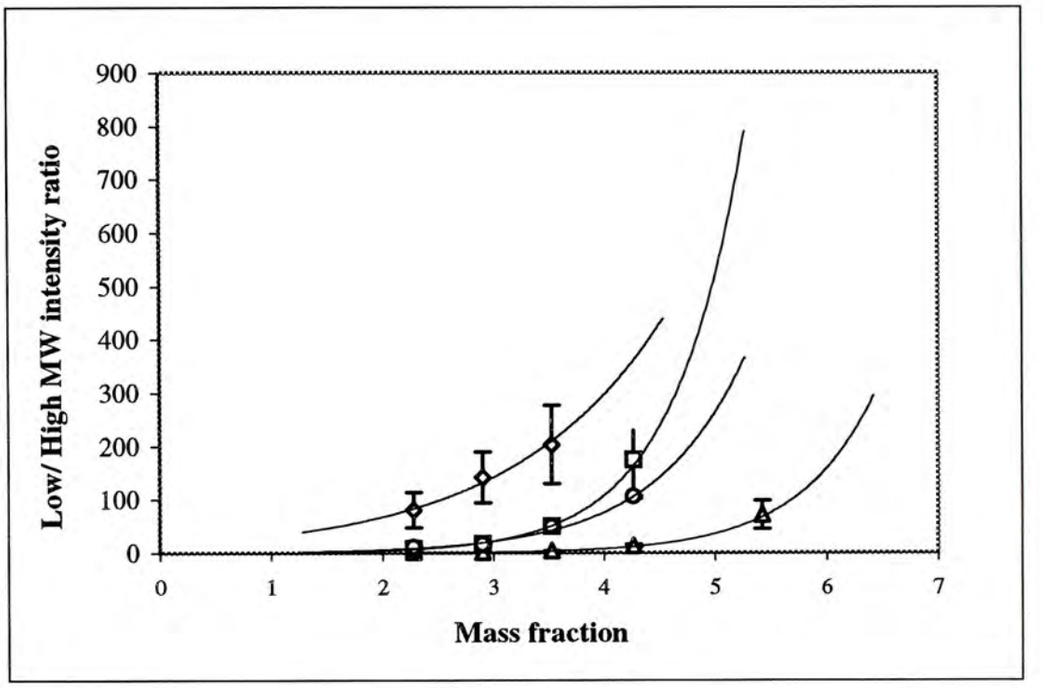


Figure 4. 5 Plots of the signal intensity ratios of the low-mass component and the high-mass component as a function of the mass ratios under different sample preparation conditions. Samples were prepared by using low-mass over high-mass layer-by-layer (◇), high-mass over low-mass layer-by-layer (△), conventional drop drying (□) and pneumatic-assisted electrospray deposition (○) sample preparation methods.

4.4 Conclusion

This report demonstrates the impact of the on-probe fractionation during the crystallization of the sample on the M_n measurement. The phenomenon of on-probe fractionation was tentatively attributed to the difference in the solubility between the low-mass and high-mass components for dispersed polysaccharides.

CHAPTER FIVE

DEVELOPMENT OF LIQUID MATRIX SYSTEMS

5.1 Introduction

In the previous Chapters, much effort has been devoted to alleviate problems associated with the use of MALDI method for the analysis of dispersed polysaccharides. Despite the development of effective sample preparation protocol for reducing molecular fragmentation during MALDI processes, there seems to be no practical solution for eliminating on-probe fractionation. The molecular weight information obtained for dispersed polysaccharides by MALDI remains inconsistent with that of GPC values. Since the on-probe fractionation originates from the different solubilities of polysaccharide molecules of different masses, sample preparation method requiring no crystallization process might provide an effective means of eliminating this problem. In this Chapter, the performance of common liquid matrix system (or matrix solution) for the analysis of polysaccharides was evaluated. Besides the absence of crystallization process, liquid matrix (or matrix solution) system was also found to provide good shot-to-shot reproducibility[89] and long sample life-span.

Unfortunately, the number of useful liquid organic compounds ever reported as matrices are limited. For MALDI instruments equipped with ultraviolet lasers, only 3-nitrobenzyl alcohol (3-NBA) and 2-nitrophenyl octyl ether (2-NPOE) have been used as matrix materials[90, 91]. However, these two materials do not exhibit significant absorption in the wavelength above 300nm. Their usage is therefore restricted to the MALDI with lasers, e.g. Nd:YAG, operating at 266nm or below. For infrared (IR)-MALDI, H_2O, glycerol, lactic acid and triethanolamine have been found useful as

matrices[92] for peptides and oligonucleotides. Nevertheless, many of these matrices need to be frozen in order to tolerate the high vacuum condition in the ionization chamber. There are no liquid organic compounds for use as matrix for nitrogen laser (λ = 337 nm), which is in turn one of the most commonly used lasers. Alternative approaches have been explored by doping laser energy absorbing materials into a non-absorbing liquid carrier to act as a matrix solution for MALDI[93]. Different formulations have been developed involving the use of small organic, inorganic and even ultrafine metal powders as laser energy absorbing materials. In all cases, liquid supports with low volatility such as glycerol and 3-NBA were used to prevent drying of the samples under high vacuum condition of the ionization chamber. More recently, Kolli and Orlando[75] proposed a rather interesting approach for formulating matrix solutions. Their approach combined two UV-MALDI (337nm) solid matrices, namely, α-CCA and 3-aminoquinoline (3-AQ) in methanol to produce a highly viscous liquid[94]. This viscous liquid was found to be a useful matrix for MALDI analysis of oligosaccharides. The signal measured was steady and long-lasting. Extending their findings, Chan et al. introduced a general formulation for converting conventional solid matrix (or other organic solid substances) into effective matrix solution[95]. In this formulation, a sufficient amount of matrix materials was dissolved in a non-volatile liquid support with the aid of suitable solubilizing agent. It was believed that stable ion-pairs exist between the matrix molecules and solubilizing agent, and are responsible for the mutual enhancement of solubility. Proteins with masses up to 66,000 Da have been successfully analyzed using matrix solutions developed using this formulation.

In this Chapter, attempts have been made to develop "tailor-made" matrix solution system(s) for analysis of polysaccharides. The effectiveness of these matrix solutions was evaluated using both fractionated (narrow distributed) and non-fractionated (dispersed) dextrans.

5.2 Experimental

5.2.1 Sample preparation

Matrix solutions were prepared according to the literature recommended procedures. In case of the well-established matrix solutions, such as α-CCA/3-AQ/glycerol and 2,5-DHB/3-AQ/glycerol, they were prepared using the reported mass ratios of 1 : 4 : 6 and 5 : 1 : 30, respectively. For other matrix solutions, they were prepared by stepwise addition of small portions of solubilizing agents (100-500 μg) into the liquid support (~ 30 mg) in the presence of saturated matrix. After each excessive addition of solubilizing agent, the mixture was sonicated to accelerate solubility equilibration. In order to avoid substantial heating effect, the mixture was thermostated under a re-circulating water-bath held at room temperature. When the added solubilizing agent failed to dissolve after prolonged sonication, the solution was deemed to have reached the solubility limit for the solubilizing agent in the presence of the matrix. The experiment was then continued by the addition of matrix into the solution until excess matrix failed to dissolve. These iterative procedures were repeated until neither more matrix nor solubilizing agent could be dissolved. The remained solids were then settled to the bottom of the vial by means of

centrifugation. The supernatant viscous liquid was then tested for effective matrix solutions. The efficiencies of these matrix solutions were evaluated by using narrow-distributed dextrans (fraction 2, see page 32 for further information).

5.3 Results and discussion

5.3.1 Formulation of matrix solutions

5.3.1.1 Development of new matrix solutions

Twelve effective MALDI solid matrices at 337 nm were selected and were converted into their corresponding matrix solutions. 2,5-Dihydroxybenzoic acid (2,5-DHB), 3-amino-4-hydroxybenzoic acid (AHBA), 2-(4-hydroxyphenylazo) benzoic acid (HABA) and 3-indoleacrylic acid (IAA) were selected because of their superior performance in the analysis of carbohydrates. Since the mechanism of action of matrix molecules in MALDI processes is not clearly understood, other matrices, such as α-cyano-4-hydroxycinnamic acid (α-CCA), sinapinic acid (SA) and nicotinic acid (NA), which are known to give excellent results in the analysis of other biological molecules were also evaluated. Figure 5.1 shows the typical MALDI mass spectra of narrow distributed dextran (fraction 2; averaged M_n ~ 2,000) using (a) α-CCA/3-AQ/Gly, (b) HABA/3-AQ/Gly, and (c) 2,5-DHB/3-AQ/Gly. In both cases, symmetric distributions of signals corresponding to the sodiated dextran molecules of different sizes were obtained. Table 5.1 summarizes the results obtained using different matrix solutions. Among the twelve matrix solutions

evaluated, only those matrix solutions derived from cinnaminic acid and its derivatives, HABA and 2,5-DHB were found to give detectable signals for the dextran sample. Among these effective matrix solutions, those solutions derived from 3-hydroxycinnamic acid, 3,4-dihydroxycinnamic acid and neat 2,5-DHB were found to have "Marangoni effect", i.e. dextran signals were only registered at the rim of the sample droplet. This peripheral effect has previously been reported for protein analysis using $Fe(CN)_6$/Gly[96] and 2,5-DHB/Gly[95,97] matrix solutions. It has been suggested that the homogeneous mixing of these matrix materials in the polar liquid supports might be responsible to cause a relatively low surface concentration of the matrix molecules, except at the rim of the sample droplet. Among the matrix solutions evaluated, α-CCA/3-AQ/Gly was found to give the highest sensitivity towards the dextran analysis.

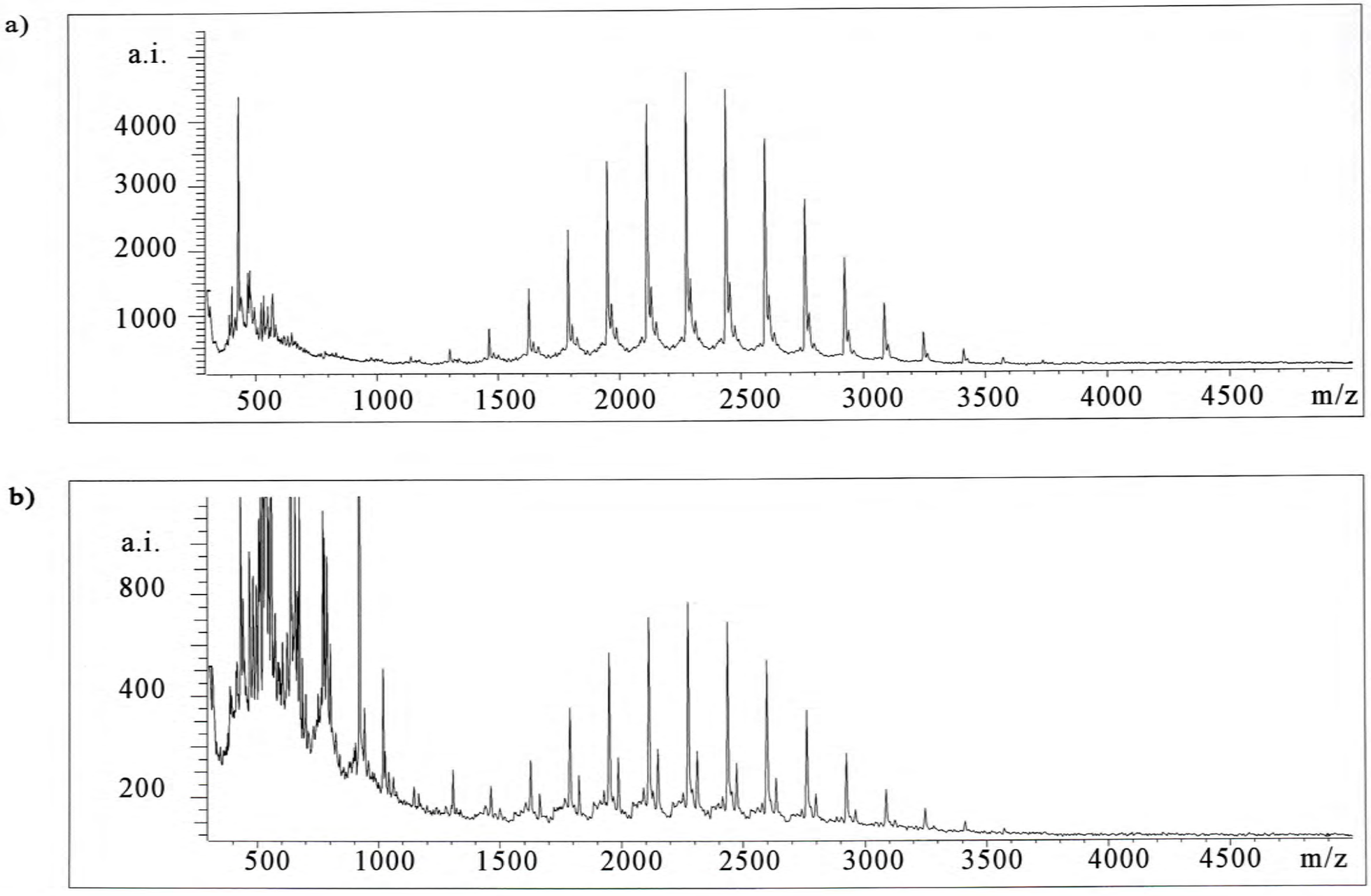


Figure 5.1 : Typical positive-ion MALDI mass spectra of fraction 2 using (a) α-CCA/ 3-AQ/ Gly and (b) HABA/ 3-AQ/ Gly matrix systems.

Table 5. 1 Summary of the matrix solutions evaluated using fraction 2 of dextran 5000. In all cases, glycerol was used as the liquid support.

Conventional matrix compound	**Solubilizing agent**	**Signal intensity (a.u.)[a]**
2,5-dihydroxybenzoic acid	--	190[b]
2,5-dihydroxybenzoic acid	3-aminoquinoline	570[b]
2,6-dihydroxybenzoic acid	3-aminoquinoline	ND[c]
3-Amino-4-hydroxybenzoic acid	--	ND
3-Amino-4-hydroxybenzoic acid	3-aminoquinoline	ND
α-cyano-4-hydroxy-cinnamic acid	3-aminoquinoline	5000
3,4-dihydroxycinnamic acid	3-aminoquinoline	150[b]
3-hydroxycinnamic acid	3-aminoquinoline	420[b]
2-(4-hydroxyphenylazo)benzoic acid	3-aminoquinoline	750
2,3,6-Trihydroxyacetophenone	3-aminoquinoline	ND
3-Indoleacrylic acid	3-aminoquinoline	ND
Nicotinic acid	3-aminoquinoline	ND
Sinapinic acid	3-aminoquinoline	ND

[a]a.u.: arbitrary unit [b]: with substantial "peripheral effect" [c] ND: not detected

5.3.1.2 New solubilizing agent development

Although the effectiveness of matrix solution depends critically on the nature of the laser absorbing matrix molecules, other components in the matrix solution such as solubilizing agent and liquid support might also influence the distribution of the matrix molecules in the solution droplet and hence the effectiveness of the matrix solution. This is particularly important for matrix solutions that exhibit substantial

"Marangoni effect". A change in the composition of the matrix solution might cause a dramatical change in the distribution of the matrix and analyte molecules within the liquid droplet. Several basic organic molecules, including ABA, ATT, DMAP and diaminonaphthalene, were evaluated as alternative solubilizing agents. Only α-CCA, 2,5-DHB and HABA matrices were investigated. Figure 5.2 shows the typical MALDI mass spectra of narrow distributed dextran (fraction 2; averaged M_n ~ 2,000) using (a) α-CCA/ABA/Gly, and (b) α-CCA/DMAP/Gly, respectively. Table 5.2 summarized the results obtained. Among all basic organic molecules tested, only ABA and DMAP were found to yield effective matrix solutions with α-CCA, DHB and HABA. In comparing to the original formulations using 3-AQ as solubilizing agent, the effectiveness of matrix solutions prepared using ABA and DMAP were found to be relatively low.

Table 5. 2 Summary of the solubilizing agents evaluated using fraction 2 of dextran 5,000. In all cases, glycerol was used as the liquid support

Conventional matrix compound	Solubilizing Agent	Signal intensity (a.u.)[a]
2,5-DHB	ABA	ND[b]
2,5-DHB	ATT	ND
2,5-DHB	DMAP	330
2,5-DHB	1,5-Diaminonaphthelene	ND
α-CCA	ABA	910
α-CCA	ATT	ND
α-CCA	1,5-Diaminonaphthelene	ND
α-CCA	DMAP	870
HABA	ABA	Insoluble
HABA	ATT	ND
HABA	1,5-Diaminonaphthelene	ND
HABA	DMAP	380

[a]a.u.: arbitrary unit [b]ND: not detected

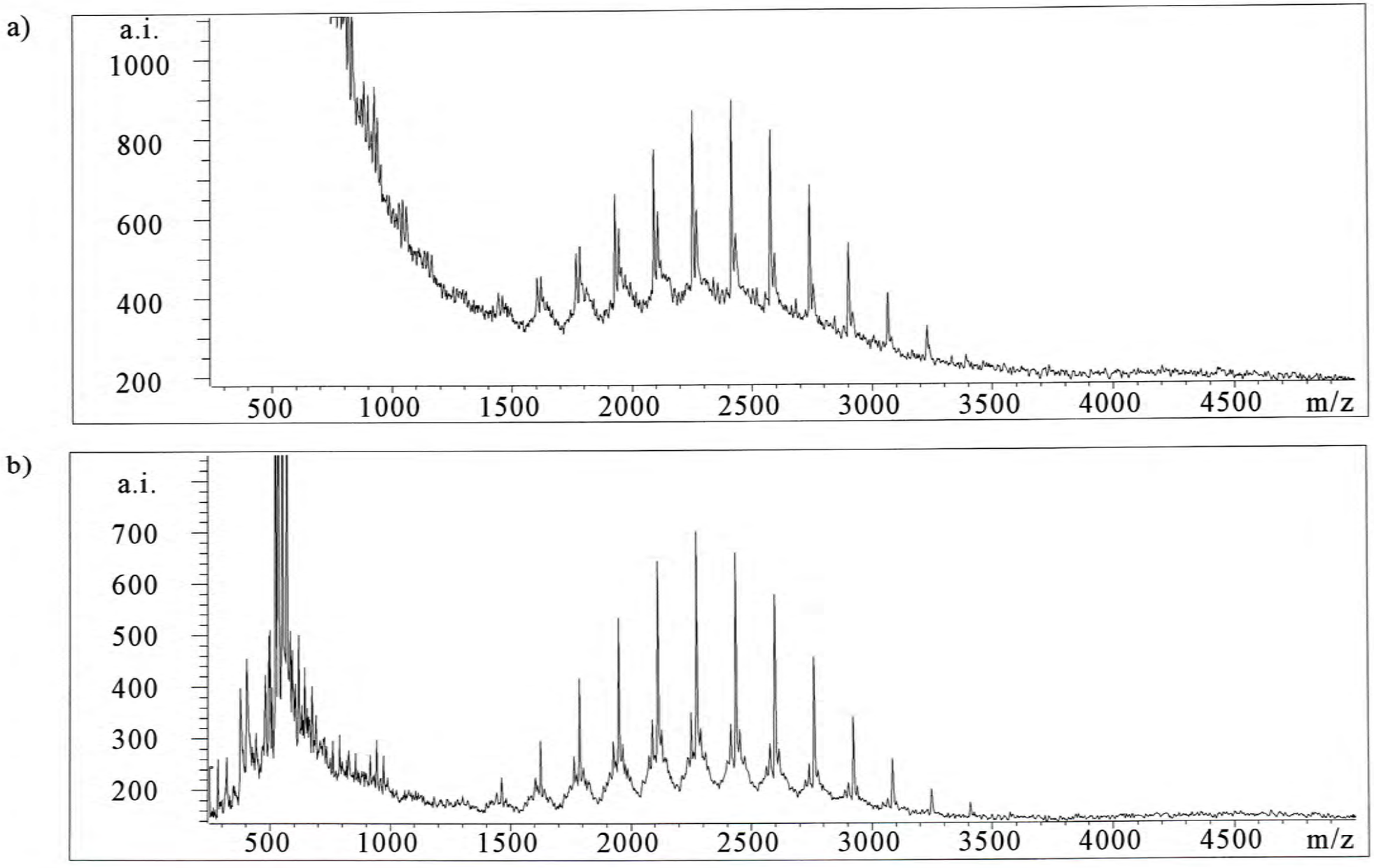


Figure 5.2 : Positive- ion MALDI mass spectra of fraction 2 using (a) α-CCA/ ABA/ Gly and (b) α-CCA / DMAP/ Gly as matrix system.

5.3.1.3 New liquid support development

In searching for alternative non-volatile liquid supports for the α-CCA and 2,5-DHB matrix solutions, conventional liquid matrices for FAB (i.e. NBA and NPOE), propan-1,2-diol (PPD) and low molecular weight polyethylene glycol (PEG) have been evaluated. Only α-CCA/3-AQ/PPD and 2,5-DHB/3-AQ/PPD has been found to give reasonable molecular ion signals for the dextran samples. Although the analyte sensitivity is lower, it is interesting to discover that the "Marangoni effect" was not observed in the 2,5-DHB/3-AQ/PPD matrix solution. This finding is empirically correlated to the reduction of the polarity of the liquid support from glycerol to PPD. Attempts to evaluate this hypothesis, PEG is used as a polarity modifier for glycerol support. Liquid supports generated by mixing various proportions of PEG and glycerol were tested. Addition of small percent of PEG into glycerol did reduce the "Marangoni effect". However, the dextran signals were significantly suppressed. Further increase in the percentage of PEG had resulted in a total suppression of the dextran signal.

Table 5. 3 Summary of the liquid support tested in the liquid matrix system

Conventional matrix compound	**Solubilizing agent**	**Liquid support**	**Signal intensity (a.u.)[a]**
2,5-DHB	3-AQ	NBA	ND[b]
2,5-DHB	3-AQ	NPOE	ND
2,5-DHB	3-AQ	PEG	ND
2,5-DHB	3-AQ	Glycerol/ NH_4OH	450[c]
2,5-DHB	3-AQ	PEG/ Glycerol (1/2)	ND
2,5-DHB	3-AQ	PEG/ Glycerol (2/1)	ND
2,5-DHB	3-AQ	PPD	2430
α-CCA	3-AQ	PEG	ND
α-CCA	3-AQ	PPD	4300
α-CCA	3-AQ	PEG/ Glycerol (1/1)(w/w)	ND
α-CCA	3-AQ	PEG/ Glycerol (1/2)(w/w)	ND
α-CCA	3-AQ	PEG/ Glycerol (2/1)(w/w)	ND

[a]a.u.: arbitrary unit [b]: ND: not detected [c]with substantial "peripheral effect"

5.3.1.4 Additives development

It has been reported that addition of suitable surfactants into the analyte/matrix mixture could modify the analyte distribution in the liquid droplet and lead to substantial improvement in the FAB analysis[98] Attempts have therefore been made to evaluate the impact of various organic molecules and surfactants on the performance of the α-CCA/3-AQ/Gly matrix system. Table 5.4 summarizes the additives used in this evaluation and the results obtained. It was found that none of the additives tested could improve the efficiency of the α-CCA/3-AQ/Gly matrix solution in the analysis of dextran sample. In fact, most of the ionizable and ionic additives showed detrimental impact on the dextran analysis. Addition of neutral additives, such as low molecular weight polystyrene (M_n 380), cetyl alcohol and decan-1-ol, have no apparent impact on the dextran analysis using α-CCA/3-AQ/Gly matrix solution. With the empirical correlation between the nature of the additives (neutral, ionizable and ionic) and their impact on the effectiveness of the α-CCA/3-AQ/Gly matrix solution, a qualitative model can tentatively be proposed to explain the observations. Ionizable and ionic additives might perturb the ion-pair structure between the matrix and the solubilizing agent and lead to a segregation of the matrix and analyte molecules. Without proper matrix isolation, the efficiency of desorption and ionization of dextran molecules becomes exceedingly low. On the other hand, neutral additives are expected to have little influence on the ion-pair structure of the matrix and solubilizing agent and are therefore expected to have no significant impact on the effectiveness of the α-CCA/3-AQ/Gly matrix solution.

Table 5. 4 Summary of the additives tested in the liquid matrix system (i.e. α-CCA/3-AQ/glycerol)

Additives	**Signal Intensity (a. u.)**[a]	**Improvement**
Cetyl alcohol	1090	-
Decan-1-ol	4010	-
Polystyrene	410	-
Cholic acid	ND	-
CTMA	ND[b]	-
$Et_4N^+ Br^-$	ND	-
$Et_4N^+ OAc^-$	ND	-
$Me_4N^+ OAc^-$	ND	-
SDS	ND	-
Retinoic acid	ND	-
Tritron X 100	ND	-
Tweenty 80	ND	-

[a]a.u.: arbitrary unit [b]: ND: not detected

5.3.2 Use of liquid matrix system

Among the various effective matrix solutions for dextran analysis, α-CCA/3-AQ/Gly was found to be the most reliable system that offers highest reproducibility and sensitivity, longer life span, and minimal mass discrimination for analysis of dispersed dextran samples. Figure 5.3 (a-c) show the typical MALDI mass spectra of narrow distributed dextran sample (fraction 4 of dextran 5,000; average M_n ~ 4,000 Da) using α-CCA/3-AQ/Gly and 2,5-DHB/NH_4F matrix systems at 100 and 50 pmol sample loadings. The resulting signal intensity of 50pmol sample in solid matrix system is higher than that of 100 pmole of sample in liquid matrix system. It is clearly demonstrated that α-CCA/3-AQ/Gly matrix solution gave a much lower spectral resolution and sample sensitivity as compared to the 2,5-DHB/NH_4F solid matrix system for analysis of dextran sample. In practice, the matrix solution system also requires higher laser fluence than the solid matrix system to effect desorption / ionization of the dextran sample. However, it was noted that α-CCA/3-AQ/Gly matrix solution induces a much lower extent of molecular fragmentation than the 2,5-DHB/NH_4F solid matrix system for analysis of dextran sample (see the insert in Figure 5.4). The reduced molecular fragmentation is tentatively associated with the higher sample pH during the sample preparation and the MALDI analysis. Finally, it was also found that matrix solution system can tolerate higher concentration of salt in the sample (data not shown). Table 5.5 summarizes the molecular weight information of narrow distributed dextran samples obtained from α-CCA/3-AQ/Gly matrix solution and 2,5-DHB/NH_4F solid matrix system. It is demonstrated that both

matrix systems provide equally reliable molecular weight information in the analysis of narrow distributed dextran samples.

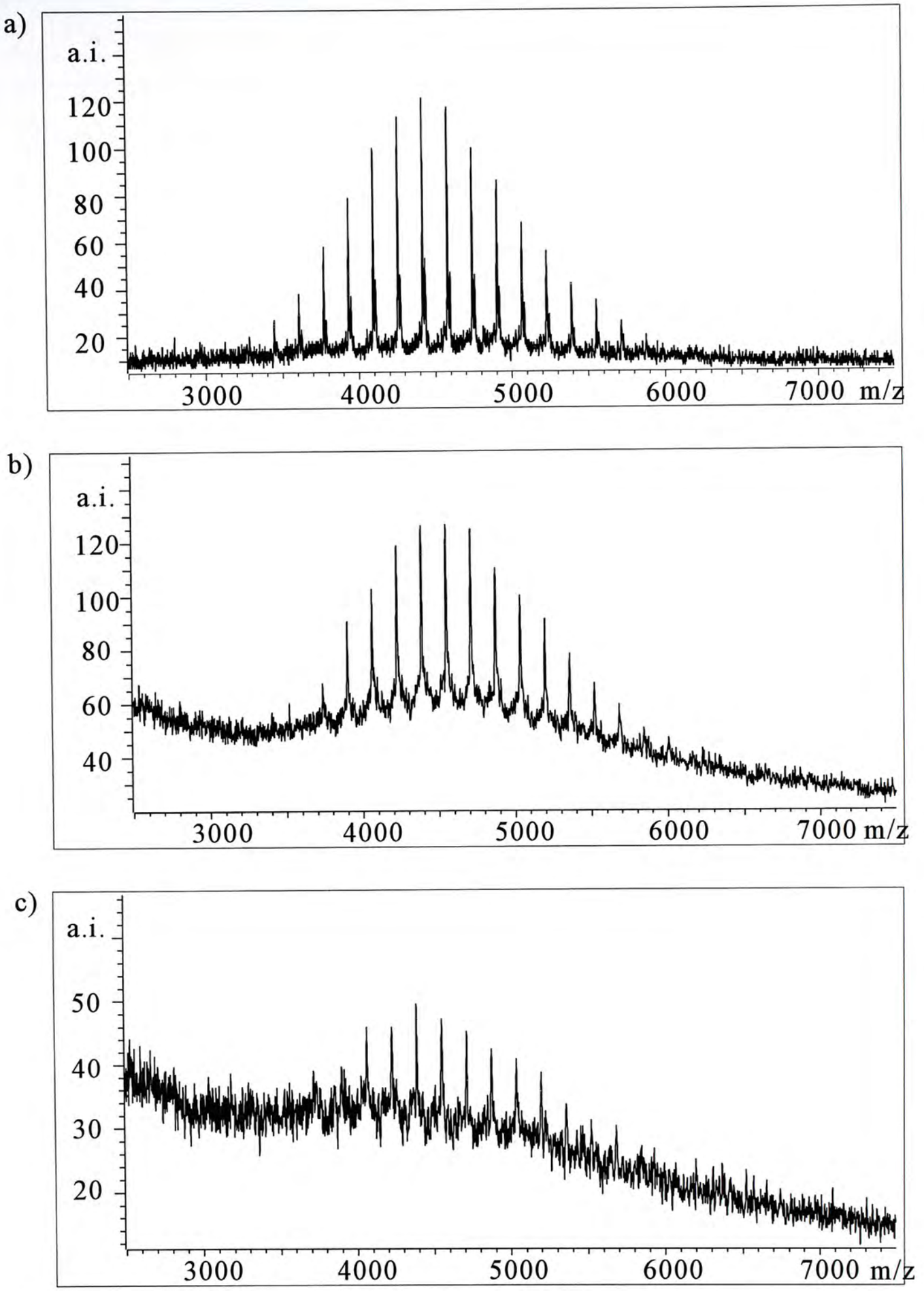


Figure 5.3 : Positive-ion MALDI mass spectra of fraction 5 using a.) 2,5 DHB/NH_4F as matrix at 50pmol/μL; b) α-CCA/ 3-AQ/ Gly at 100pmol/μL and c) at 50pmol/μL.

Table 5. 5 A comparison of molecular weight information obtained from solid and liquid matrix systems for 2 narrow distributed dextran samples.

	Sample A		**Sample B**	
	Solid matrix	*Liquid matrix*	*Solid matrix*	*Liquid matrix*
M_n	4,540	4,720	8,570	8,700
M_w	4,620	4,790	8,670	8,800
P.D.	1.0	1.0	1.0	1.0

5.3.3 Analysis of dispersed dextran

Although α-CCA/3-AQ/Gly matrix solution has a lower sensitivity and produces mass spectra of lower resolution, it does provide reliable molecular weight information of narrow distributed dextran samples. Since the sample prepared by using matrix solution involves no crystallization process, it is worthwhile to assess the effectiveness of the matrix solution system in analyzing dispersed dextran samples. Figure 5.4 shows the typical MALDI mass spectra of dextran 1,000, 5,000 and 12,000 using α-CCA/3-AQ/Gly matrix solution. Table 5.6 summarizes the molecular weight information obtained. It was found that the use of matrix solution system does not impose dramatic improvement in the analysis of dextran 1,000. The mass range covered in both solid matrix and matrix solution systems were similar. In comparison with the values obtained from GPC method, molecular weight information (i.e. M_n and M_w) obtained using α-CCA/3-AQ/Gly matrix solution was slightly overestimated and the polydispersity (P.D.) and the M_p value were

underestimated. In contrast to the 2,5-DHB/NH_4F matrix, it was found that the M_n measured using α-CCA/3-AQ/Gly matrix solution is more compatible with the GPC for even for relatively high mass dextran samples, i.e. dextran 5,000 and 12,000. The peak distribution is more symmetric.

Table 5. 6 MW information determined by MALDI-TOFMS in α-CCA/ 3AQ/ Gly.

	Dextran 1,000	**Dextran 5,000**	**Dextran 12,000**
M_n	1,500	5,640	8,940
M_w	1,710	8,360	12,920
P.D.	1.1	1.5	1.4

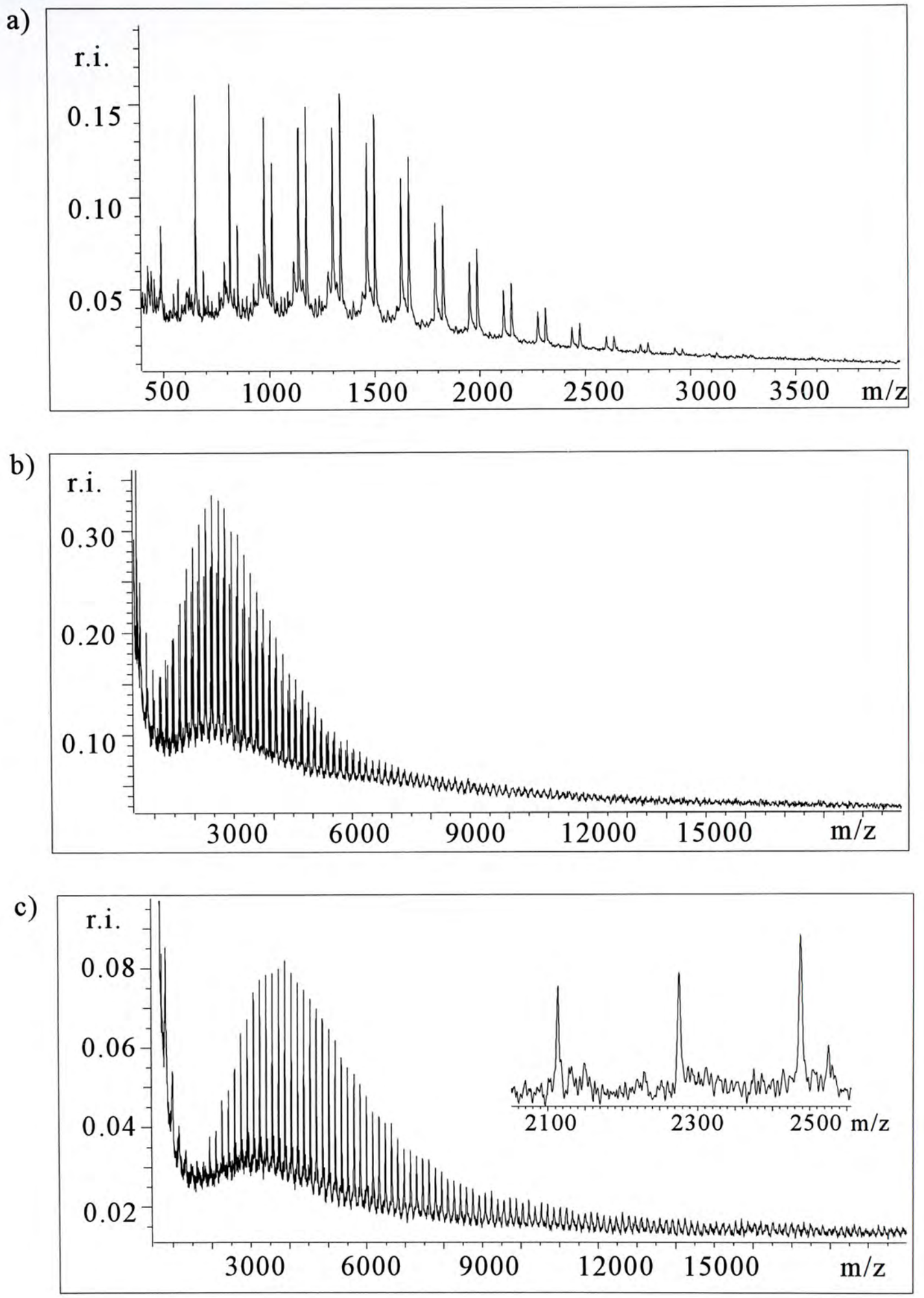


Figure 5.4 : Positive-ion MALDI mass spectra of dispersed (a) dextran 1,000, (b) dextran 5,000 and c) dextran 12,000 using α-CCA/ 3AQ/Gly matrix system.

5.4 Conclusion

It has been demonstrated that matrix solution is more compatible for the analysis of dispersed dextran samples. By reducing the extent of molecular fragmentation and on-probe fractionation, a more reliable M_n (M_w) was obtained for dispersed dextran samples using α-CCA/3-AQ/Gly matrix solution.

6.1 Conclusion

Accuracy, precision, fast analysis and easy operation are the pronounced advantages of MALDI-TOFMS. After ten years development, its application becomes more popular. It is moving from research analysis to industrial uses, from single protein to proteomic analysis including molecular weight determination as well as structural analysis. On the other hand, its application on polymer science was only limited to molecular weight determination of narrow polymer distribution. Molecular weight measurement of dispersed polymers by MALDI-TOFMS offers many advantages over GPC especially for the analytes which cannot be detected by UV detector, such as polysaccharides. Nevertheless, many researchers have preformed different experiments in attempts to understand the reasons of the failure. Much emphasis has been placed on the study of synthetic polymers, such as polystyrene (PS) and (PMMA) rather than biopolymers.

Polysaccharide was the target analyte and dextran was used as the test. In the present study, the failure of this labile biopolymer analysis by MALDI-TOFMS was investigated, and solution was proposed then. In the analysis of dispersed polymers, the high-mass molecules were not detected. Since the problem was due to the failure of either desorption/ ionization or detection. First, the search for the better sample preparation to enhance desorption was performed and the problem of sample fragmentation was also evaluated. In the experiments, the relation of fragmentation and the molecular weight was clearly identified. The extent of fragmentation of

dextran molecules was proportional to the molecular weight. The extent of fragmentation becomes significant until there is a failure to detect the intact ions of higher mass molecules. This acid catalyzed cleavage via the glycosidic linkage which was induced by the presence of strong acid, 2,5-DHB. As it leads to the difficulty in detecting the high mass molecules, it was logical to predict that it is a reason that leads the result inconsistent with the GPC result. Although, an additive with high proton affinity, ammonium fluoride, was successfully applied to eliminate fragmentation, it does not impose significant improvement in analysis of dispersed sample. In other words, it was not the critical factor that restricted the application to dispersed polymer analysis.

Second, the effect of sample preparation was evaluated by applying different layering preparation methods. It was noted that the differential solubility across a range of molecular weights was critical to the molecular weight measurement. According to the result of analysis, it was proposed that the difference in solubilities between the low-mass and high-mass components for dispersed polymers might be large enough to induce an enhancement of low-mass components at the surface for the crystals during sample crystallization in the drop-drying method.

Thirdly, as differential solubility was natural physical property, liquid matrix, a method free from crystallization, was applied, thus improvement was expected. With tedious trial and error experimentation in selection for a better matrix system, α-

CCA/3-AQ/ Gly was chosen. It was shown that the use of a liquid matrix system was able to lower the extent of fragmentation. In addition, the mass range covered was slightly expanded and the molecular weight information was comparable to that obtained from GPC measurement. From these results, it is proven that that liquid matrix system can also be successfully applied to the analysis of polysaccharide and molecular weight information was reliable in contrast to the result obtained from solid matrix.

In conclusion, all of these experimental results support the conclusion that sample preparation, such as preparation method and matrix system selection, is a critical issue on the molecular weight determination of polysaccharide. In addition, the problem of on-probe fractionation seems unable to be solved. Fortunately, the liquid matrix system is another substitute to replace of solid matrix system. In the use of liquid matrix, improvement was achieved in term of life span of the spot, capability of reducing fragmentation and also the result of molecular weight determined. All of these results support the conclusion that the development of liquid matrix systems should be a new research direction that could further expand the application of MALDI-TOFMS to wider areas.

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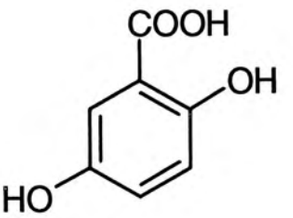
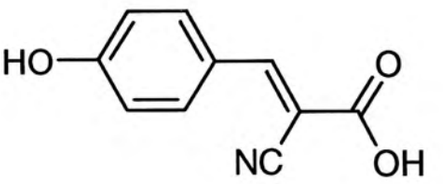
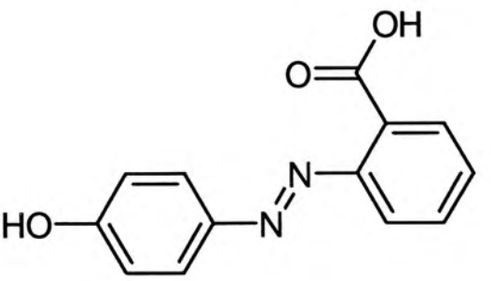
Appendix 1: Chemical structure of matrices



2,5-Dihydroxybenzoic acid

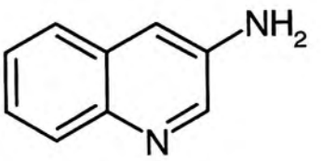


α-Cyano-4-hydroxy-cinnamic acid

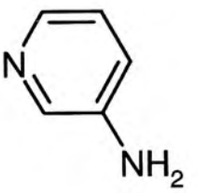


2-(4-Hydroxyphenylazo) benzoic acid

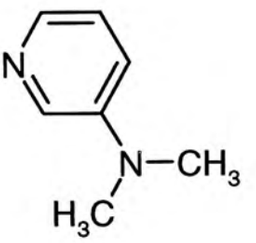
Appendix 2: Chemical structure of solubilizing agents



3-Aminoquinoline

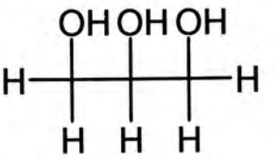


3-Aminopyridine

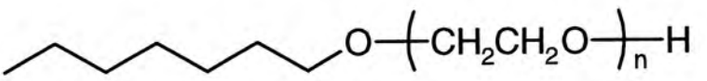


3-Dimethylaminopyridine

Appendix 3: Chemical structure of liquid support

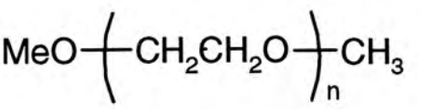


Glycerol

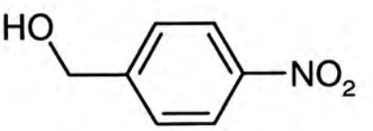


n=9-10

Octyl-polyoxyethylene

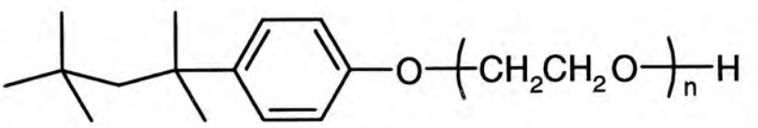


Polyethylene glycol

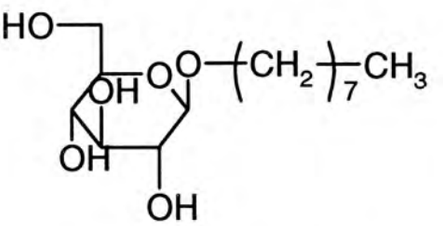


3-Nitrobenzyl alcohol

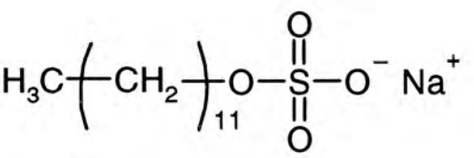
Appendix 4: Chemical structure of additives



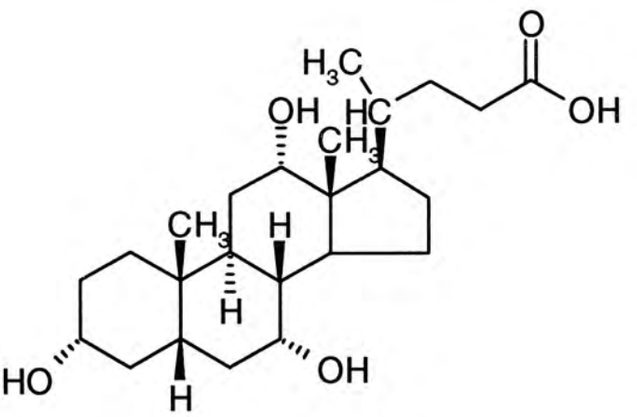
Triton X



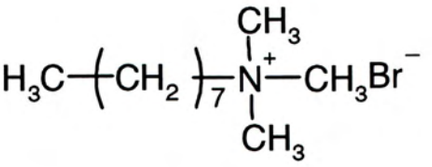
β–Octylglucosides



Sodium dodecyl sulphate



Cholic acid



Cetyl trimethylammonium bromide